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### CORRECTION.

The accompanying paragraph is to replace the first paragraph on page 175, Vol. LV, No. 2, February, 1923. The slip is arranged to be pasted over the place occupied by this paragraph at present.

### CORRECTION

On pages 394 and 395, Vol LV, No 3, March, 1923, the word "fowl" should be employed instead of the term "pigeon" in reference to the work of Eijkman, van Hoogenhuijze, and Derks.

### CORRECTION.

On page 771, Vol LVI, No. 3, July, 1923, first paragraph in small type, referring to Warburg's concentration unit, should conclude "*and also the  $Cl'$  and  $HCO_3'$  concentrations*" instead of "*but not the concentrations of the other solutes.*"





# THE PRESERVATION OF BLOOD FOR CHEMICAL ANALYSIS.

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(Received for publication, July 5, 1923.)

This study has been made for the purpose of preserving those chemical constituents which are usually determined in a routine analysis of blood for clinical purposes, so that an immediate analysis is not necessary and blood samples may be kept under

In Experiment 2 in which sodium sulfate was the salt under Dr. investigation results of a somewhat different nature were obtained. In the case of the sulfate radical a maximum increase of 76.9 mg. per 100 cc. of serum (an increase of about 2,480 per cent) was found after an absorption period of 210 minutes; and 27 mg. per 100 cc. of whole blood (1,350 per cent increase) in during the same time, while the sodium content of the serum increased from 310 to 338 mg. (9 per cent) and the sodium content was of whole blood from 216 to 218 mg (0.9 per cent).

A review of the literature shows that but little work has been reported on the preservation of blood for chemical analysis. Denis and Aldrich (1) report that formaldehyde is an excellent preservative for sugar and give data which show that blood sugar remains constant for at least 96 hours. Oppenheimer (2) has been able to preserve blood from slaughtered animals for 5 to 6 days with sodium acid sulfite. Salkowski (3) was able to preserve blood for about 5 days with large concentrations of boric acid and salicylic acid, formaldehyde, and with allyl alcohol in concentrations of 0.5 to 0.6 per cent (4). No analysis of the chemical constituents was made by these workers, since they were interested only in preserving blood for food purposes.

Falk, Baumann, and McGuire (5) have reported studies made on the chemistry of spoiled meat which would seem to indicate

that the substances investigated were remarkably constant for some time. They found that unpreserved meat kept for 10 to 11 days at 5–15°C. showed a very decided increase in ammonia content, and that if the ammonia was first removed or a correction made for the ammonia value, the non-protein nitrogen, creatine, creatinine, and purine nitrogen remained practically unchanged. These authors also found that many of the common bacteria, studied individually, had no effect on the creatine, creatinine, purine nitrogen, or non-protein nitrogen, when correction was made for ammonia content.

That at least some of the constituents are very constant in blood is indicated by the fact that in our serological department whole blood kept under ordinary sterile conditions in midsummer without the addition of any preservative or chemical substance has been found to give a perfectly reliable correct Wassermann reaction after 10 days standing at ordinary temperatures. In the present study the possibility of bacterial action, autolysis or heterolysis, and chemical changes, has been constantly kept in mind. The substance used as a preservative should cause no interference with, nor chemical changes in, the reagents used in the various tests; should not be alkaline or cause the blood to become more alkaline in reaction since each of the determined constituents is more labile even in slightly alkaline solutions; should not combine with any element of the blood, and thus be removed from active solution. Such substances as chloroform, phenol, sodium sulfite, sodium cyanide, and copper and mercuric salts, were given little consideration.

#### EXPERIMENTAL.

The system of blood analysis, as outlined by Folin and Wu (6), was used in all tests at first, and later the method of Benedict for uric acid was substituted for the uric acid method of Folin and Denis.

All precipitations of proteins were made with sodium tungstate and sulfuric acid. Tests made upon blood of high nitrogen content, and even bloods which had stood for at least 2 weeks with and without the addition of preservatives gave practically the same non-protein nitrogen content when precipitation was made by the

above method, or with 25 per cent *m*-phosphoric acid, or with 10 per cent trichloroacetic acid, or with alumina cream (7).

The urease method was used in all determinations of urea, and at first the ammonia was removed by distillation, but later the aeration process was used. Air was drawn through a train of wash bottles before entering the aeration apparatus. Suction was employed, rather than pressure, since any slight leak would not tend to cause loss of ammonia, as would be the case if a blast of air was used for aeration. 15 minutes aeration are usually recommended, yet in solutions of pure urea 30 to 40 minutes aeration were found necessary. In all determinations, therefore, 1 hour was always allowed for the aeration of ammonia. Frequent comparisons were made of the aeration method with the distillation method for the recovery of ammonia and very comparable results were obtained. In the latter part of the work the urea determination was made with whole blood rather than with blood filtrate.

The method of Folin and Denis (8) for uric acid was used at first, but was soon replaced by the method of Benedict (9) for uric acid, since a larger number of analyses could be made in the same time and the results were equally as satisfactory. In both methods a precipitate appeared at times before color comparisons could be made. This was noticed especially in those bloods which were kept for more than 10 days before analysis. As a rule, the uric acid in six to eight blood filtrates was determined at the same time, and in case a precipitate appeared in any tube before a reading could be made, then that determination was repeated separately.

For creatine and creatinine determinations the methods as outlined by Folin and Wu (6) were used, and all the picric acid was recrystallized several times (10), and conformed to the standards of purity given by Folin and Doisy (11).

For the sugar determination the method of Folin and Wu (12) was used throughout, because of the ease with which many samples could be analyzed simultaneously and because it was felt that, in principle at least, this method gave a more correct value for sugar than any other method (13, 14).

The blood used in all this work was drawn from patients in the mornings after breakfast, in order to obtain high values for

all the substances investigated. Immediate analysis of the blood obtained was made, in all series of determinations, as a control. In most of the work which required more than 15 cc. of blood, the blood from several patients was drawn, well mixed, and an analysis made in duplicate on the mixed blood.

The work was divided into three main divisions. (1) Blood was drawn under aseptic conditions and a measured amount placed into sterile bottles which contained known amounts of the substance under investigation as a preservative. Practically every substance commonly used as a preservative was used in this preliminary trial. The stoppered bottles of blood were allowed to stand for 6 days at room temperature and an analysis was made. This preliminary investigation of common preservatives was made four times and where any preservative investigated seemed to maintain any of the constituents reasonably constant, it was used as outlined in the following step. (2) Blood was drawn under aseptic conditions, well mixed, and 5 cc. were placed in a series of sterile bottles which contained a known amount of the preservative that had given even fair results under the condition as outlined in the first step. As a rule six to nine bottles constituted a series and each bottle contained exactly 5 cc. of blood and the same amount of the same preservative. Each day a bottle was chosen at random from the series, the blood diluted, and the blood proteins were precipitated in that bottle. At times varying concentrations of the preserving material were used and the results of each series of determinations were duplicated at least four times before a possible preservative was rejected or retained for further study. (3) 50 to 100 cc. of mixed blood were put into non-sterile bottles which contained those preservatives chosen from Tests (1) and (2) above. These bloods were allowed to stand for several hours exposed to the air before the bottle was stoppered. Each day 5 cc. of blood were drawn from each bottle and an analysis was made. The time of complete hemolysis was noted as closely as daily observation permitted, and some of the last portions of blood were kept under laboratory conditions for months or until the blood had become badly decomposed, and putrefaction was shown by the presence of large quantities of ammonia.

This same procedure, as outlined in the third step, was later followed, with those preservatives which were finally thought to preserve effectively the constituents investigated, upon (a) normal bloods, (b) bloods to which known amounts of urea, uric acid, creatinine, creatine, and sugar had been added, and (c) pathological bloods. Some of these later series were kept in the laboratory at temperatures of 18–29°C. and some series in the incubator at 37–39°C. Most of the results of these studies on the preservation of the pathological specimens of blood will be reported in a separate clinical paper. It is sufficient to state that the results obtained with pathological bloods approximate very closely in all respects the results shown in the cases given below and the results shown in "loaded bloods."

No preserving substance could be found which, when used alone, would preserve a sample of blood for 4 or 5 days and maintain the values for all the investigated substances unchanged. In general, metallic salts, in concentrations up to 1.5 per cent were found to have little effect in preserving sugar, urea, and uric acid. Alkaline substances, such as sodium cyanide, caused complete disappearance of sugar, urea, and uric acid in a very few hours. Such substances as benzoic acid, boric acid, zinc chloride, etc., in concentrations up to 2 per cent did not prevent loss of sugar in the first 2 days, nor inhibit a steady rise in non-protein nitrogen value. Substances such as ether, lysol, phenol, benzene, toluene, acetone, etc., were found to be very ineffective as a preservative, especially with sugar and non-protein nitrogen. Many substances, such as chloroform and acriflavine, gave such interference with the color determinations that other methods of analysis were used. Formaldehyde gave varying results when used alone as a preservative or in combination with other substances. A very decided increase in sugar values was frequently noted within the first few days, and at times the sugar value became zero within 48 hours. In practically all cases where formaldehyde was used as a preservative, after 5 to 6 days the value for sugar had become zero or so low that the values were unreliable when compared to the control values. This was noticed especially in bloods of very high sugar content.

In general, for all preservatives tried, it was found that when a single substance was used as a preservative the non-protein

nitrogen content increased rapidly and continuously. The value for urea, after the blood had stood for 6 days, could only be determined after removal of ammonia with permutit, or by evaporating the blood filtrate to small volume after it had been made alkaline to phenolphthalein with sodium carbonate. The ammonia was generally removed by permutit extraction before the urea was determined. The value for urea in 6 days was generally very much lower than the control, and in many cases was zero.

The uric acid content remained remarkably constant in most cases where any preserving action was evident for at least 4 days and gradually disappeared in 6 to 10 days.

The creatinine and creatine values were very constant throughout all the work, and even in unpreserved blood, kept for 6 days, the values for these substances were practically the same as the values of the controls. No case was found in all the work where a complete absence of these substances was indicated; that is, where the sample of blood filtrate, treated with alkaline picrate, gave the same colorimetric readings as the alkaline picrate of the same dilution. Creatine and creatinine are remarkably stable compounds or their decomposition products have the same quantitative color production as the original substances. This same stability of creatine and creatinine has been observed by Falk, Baumann, and McGuire (5) in muscle tissue which had been kept for 10 days and had decomposed to some extent.

The value for sugar in the blood is usually regarded as the most labile substance of the blood. It was felt that any preservative which did not maintain the value for sugar unchanged for at least 10 days could not be considered as a good general preservative for blood. More importance was, therefore, attached to the sugar content throughout this series of experiments than to any other single constituent.

In summing up the preliminary work, as outlined in Steps (1) and (2), it was found that no single preserving substance could be relied upon to maintain unchanged the values for non-protein nitrogen, urea, uric acid, and sugar for a period of 48 hours; creatinine and creatine remained constant for several days in unpreserved blood and in blood containing many of the common preservatives.

An attempt was then made to combine two or more of the preservatives which had seemed most efficient. In all the preliminary work sodium or potassium oxalate was used to prevent the clotting of blood, but early in the work when calcium, barium, and strontium salts were used for preservation, sodium fluoride was used as an anticoagulant. Several series of experiments were made to study the changes caused by decomposition and putrefaction of the blood in the presence of sodium fluoride, as had been done with oxalated blood. Several samples of blood in a series of eight bloods, kept in sterile bottles for 6 days, showed excellent preservation of sugar, uric acid, creatinine, and creatine. When the same experiment was repeated but one blood sample in the series of eight gave values approximating those of the controls. The concentration of sodium fluoride was 0.02 gm. for 10 cc. of blood and when this concentration was increased to 0.1 gm. for 10 cc. of blood the sugar content in four of the bloods was the same as that of the controls, and in four of the bloods the content was zero. The urea content remained unchanged in several of the bloods. It was thought that in those cases where little preservation of the blood was indicated sterile conditions had not been maintained and (a) the loss of constituents was due to bacteria, yeasts, molds, etc., or (b) the increase of alkalinity due to putrefaction made the constituents more labile. Later work would seem to indicate that the second assumption was incorrect, for when sodium fluoride was used in combination with organic and mineral acids, no better results were obtained than with sodium fluoride alone.

Since sodium fluoride gave better results in preserving blood specimens than any other single substance used, combinations of sodium fluoride and many other substances were tried. After many preliminary trials a combination of sodium fluoride and thymol was found to give most satisfactory results. Sodium fluoride or thymol alone will not preserve those blood constituents investigated for a period of 5 to 6 days, while a combination of sodium fluoride and thymol is apparently very efficient.

Sodium fluoride and thymol have but little effect upon the reagents used in each analysis and cause no interference with methods. Sodium fluoride and thymol were used in concentrations ten times that recommended for preserving blood samples



and no interference with methods was found. A large concentration of thymol produces a slightly deeper blue color in a water blank test with Benedict's uric acid reagents, but this apparently does not alter the values for uric acid as shown later in normal blood, "loaded blood," and pathological blood specimens when compared to the same bloods drawn into sodium oxalate. The hydrofluoric acid released in the non-protein nitrogen determination does not affect the test-tubes, and some of the Pyrex test-tubes which have been used hundreds of times show no traces of hydrofluoric acid etching. Sodium fluoride as purchased was free from ammonia and 1 gm. treated with 5 cc. of Nessler's solution gave no color.

A series of determinations was made to determine the optimum concentration of sodium fluoride and thymol for the preservation of blood samples. It was found that if 0.01 gm. of sodium fluoride and 0.001 gm. of thymol was used for each cubic centimeter of blood, a sample of blood could be kept for at least 10 days before analysis. The value for sugar, uric acid, creatine, and creatinine remained constant, while the non-protein nitrogen and urea value increased slightly. Gad-Andresen (15) states that in muscle, usually within 4 to 5 hours, all the urea is transformed into ammonia, while Medwedew (16) showed that oxalated blood kept under aseptic conditions for 24 hours showed very decided increases in ammonia content. That the increase in non-protein nitrogen value was not due to ammonia from the decomposition of urea, was indicated by the fact that when ammonia was removed from blood filtrate by permutit extraction, the urea value was decreased but slightly. The amino-acid content of preserved bloods was then studied in an effort to account for this steady rise in non-protein nitrogen value and the recent method of Fohn (17) for amino-acid in blood was used. When the blood filtrate was extracted with permutit the amino-acid value remained almost constant for 10 days, and showed but a slight increase.

When the above concentration of sodium fluoride and thymol was used, the bright red color of well shaken, freshly drawn blood was maintained for at least 6 days, and frequently for 10 to 14 days. When carefully drawn and preserved, a straw-colored serum was obtained, after the blood had been centri-

TABLE I

Days	0	1	2	3	4	5	6	7	8	9	10
<b>A.</b>											
Sodium oxalate, 0.002 gm. per 1 cc. blood.	27 3	29 3	36 3	42 4	45 0	51 3	55 4	62 7	71 0	76 2	85 3
Urea	14 3	16 2	18 9	23 0	24 7	26 1	28 6	30 0	32 3	36 4	39 5
Uric acid . . .	3 79	4 02	3 60	3 48	3 50	3 25	3 30	3 05	3 00	2 71	2 10
Creatinine .	1 50	1 47	1 51	1 48	1 38	1 35	1 40	1 30	1 33	1 30	1 34
Creatine	5 22	5 35	5 10	5 00	4 90	5 16	5 12	4 82	4 75	4 80	4 55
Sugar	95 2	78 2	20 2	0	0	0	0	0	0	0	0
<b>B.</b>											
Sodium fluoride, 0.01 gm. per 1 cc. blood.	32 7	35 2	35 6	36 2	35 4	35 2	38 1	41 0	40 0	41 2	42 6
Urea	13 7	13 8	14 3	15 5	15 9	15 0	15 5	15 9	17 2	17 8	18 7
Uric acid	3 59	4 00	3 87	3 80	3 85	3 87	3 55	3 40	3 21	2 73	2 20
Creatinine .	1 44	1 50	1 55	1 40	1 44	1 47	1 37	1 46	1 47	1 51	1 41
Creatine	4 34	4 10	4 40	4 41	4 27	4 08	4 21	4 02	3 97	4 09	3 95
Sugar	135	137	134	127	92	33	0	0	0	0	0
<b>C.</b>											
Thymol, 0.002 gm per 1 cc. blood and sodium oxalate, 0.002 gm. per 1 cc. blood	25 3	25 9	29 2	37 3	39 2	46 1	49 9	60 0	72 1	75 2	76 1
Urea	13 4	13 9	13 0	16 7	15 9	17 0	16 8	18 1	20 2	22 3	27 7
Uric acid	3 08	3 18	3 00	3 12	3 20	3 03	2 92	2 86	2 91	2 76	2 75
Creatinine . . .	2 24	2 32	2 33	2 22	2 33	2 31	2 27	2 25	2 10	2 17	2 08
Creatine . . .	7 45	7 42	7 58	7 52	7 51	7 25	7 32	7 19	7 07	7 12	6.94
Sugar	91 3	80 2	37 6	0	0	0	0	0	0	0	0

The above results are expressed in mg. per 100 cc. of blood. Non-protein nitrogen and urea values are uncorrected for ammonia content.

TABLE II

Average results of 50 determinations      Concentration of sodium fluoride 0.01 gm per 1 cc. of blood      Concentration of thymol 0.001 gm per 1 cc. of blood      Non-protein nitrogen and urea values are uncorrected for ammonia content.      Results expressed as mg per 100 cc of blood

Days	0	1	2	3	4	5	6	7	8	9	10	11	12	13	Mg per 100 cc added
A Normal blood.															
Non-protein nitrogen.	36.3	36.1	37.0	39.0	38.1	38.7	39.5	39.2	40.1	42.2	42.9				
Urea . . . . .	17.8	16.9	16.8	17.2	17.5	18.0	18.3	18.0	18.5	19.2	19.0				
Uric acid . . . . .	3.55	3.58	3.53	3.47	3.52	3.58	3.50	3.46	3.51	3.45	3.42				
Creatinine . . . . .	1.32	1.29	1.30	1.35	1.31	1.30	1.36	1.30	1.29	1.33	1.30				
Creatine . . . . .	4.35	4.27	4.39	4.29	4.20	4.31	4.39	4.36	4.35	4.26	4.37				
Sugar . . . . .	127	126	129	131	130	129	128	129	131	126	128				
B Blood to which was added a known amount of each substance investigated															
Non-protein nitrogen.	64.5	66.4	65.3	67.7	66.4	66.7	68.2	69.1	67.5	69.2	69.5	68.4	71.9	72.8	
Urea . . . . .	38.4	39.8	41.0	41.2	40.8	42.1	42.9	45.2	44.3	45.6	46.1	46.8	46.1	47.2	60
Uric acid . . . . .	9.45	9.30	9.18	9.41	9.16	9.32	9.10	9.42	9.32	9.08	9.17	9.02	8.96	9.20	6
Creatinine . . . . .	7.32	7.10	7.54	7.40	7.20	7.31	7.18	7.60	7.16	7.38	7.26	7.40	7.65	7.32	6
Creatine . . . . .	13.4	14.3	13.7	13.9	13.4	14.2	14.3	14.0	13.8	13.2	13.9	13.5	13.4	13.1	6
Sugar . . . . .	326	332	318	325	317	324	322	322	326	320	317	327	318	321	200

Days . . . . .	0	1	2	4	6	7	10	12	14	Mg per 100 cc added
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## C. Normal blood kept at 37-39° during experiment

Non-protein nitrogen	35.7	35.6	37.2	38.7	44.3	46.5	49.5	53.4	57.1	
Urea.....	16.0	15.1	16.2	17.1	18.8	19.2	28.3	31.8	33.3	
Uric acid..	3.52	3.55	3.40	3.29	3.25	3.17	2.99	2.79	2.88	
Creatinine...	1.33	1.39	1.29	1.53	1.66	1.60	1.51	1.63	1.68	
Creatine .....	5.09	5.15	4.88	5.17	5.00	5.20	4.90	5.15	4.98	
Sugar....	106	103	105	100	107	103	107	104	101	

## D. Blood to which known amounts of each substance were added Blood kept at 37-39° during experiment.

Non-protein nitrogen	61.5	59.2	62.7	60.6	65.2	64.3	74.0	75.4	78.1	60
Urea.....	37.1	38.2	37.3	39.6	40.7	41.6	44.3	45.1	46.2	6
Uric acid .	9.18	9.45	9.37	9.76	9.80	9.71	9.36	8.93	9.01	6
Creatinine .	7.62	7.50	7.69	7.43	7.45	7.39	7.39	7.61	7.28	6
Creatine .....	15.6	16.2	15.6	15.9	16.1	16.2	16.0	15.6	15.5	6
Sugar .....	308	312	269	300	295	304	308	301	299	200

fuged, after 4 to 6 days. Frequently, this serum had a faint pink color, indicating slight hemolysis. Concentrations of sodium fluoride and thymol above those recommended were apparently not necessary for preservation of blood, but in no way interfered with the analysis. The blood was hemolyzed in much less time with higher concentrations of the preserving materials, and frequently the blood assumed a jelly-like appearance, but constant values were obtained for those substances investigated.

Table I gives the average results from eight series of determinations. A comparison of the oxalated blood with that of blood treated with sodium fluoride shows that the non-protein nitrogen values do not increase as rapidly as in the oxalated blood, and the disappearance of sugar is delayed for several days. Thymol seems to have as little preserving value as sodium oxalate.

From Table II it is apparent that the non-protein nitrogen and urea values rise more rapidly when preserved blood was kept at 37° than at ordinary laboratory temperatures. The uric acid value remained constant for at least 10 days, while at the end of 2 weeks creatinine, creatine, and sugar values showed no depreciation at all. The blood had been exposed to the air in non-sterile bottles and the results of each series of analyses were duplicated many times.

The cases in Table III are shown because of the high values of the nitrogenous constituents. A day by day analysis was not possible since only a relatively small quantity of blood was obtained in each case. It will be seen that the determinations for non-protein nitrogen and urea show much larger individual variations than in the former tables. Even in determinations where the blood filtrates were diluted so that the pathological constituents had about the same concentrations as in normal bloods, still duplicate analyses showed no better individual agreement. The analysis, however, seems to show a gradual rise in the non-protein nitrogen and urea values, as already indicated in normal and "loaded blood." It may be concluded that even after 10 days standing an analysis of the blood would have the same clinical significance as an analysis made soon after the blood was drawn.

TABLE III.

*Preservation of Pathological Blood.*

Mg. per 100 cc. of blood.

Days	Case 19, S J					Case 32, M S.				
	0	3	6	8	10	0	2	5	7	10
Non-protein nitrogen ..	176.4	152.3	185.2	198.1	208.6	81.1	90.6	75.2	92.6	96.2
Urea.....	80.1	85.6	76.1	90.2	93.2	27.8	29.9	31.7	32.1	32.3
Uric acid.....	11.55	11.18	11.68	11.10	11.32	8.97	8.75	8.89	8.99	8.83
Creatinine.....	9.75	9.40	9.29	9.68	9.45	3.44	3.60	3.40	3.52	3.47
Sugar.....	222	210	217	208	214	112	106	110	114	109

Concentration of sodium fluoride 0.01 gm. per 1 cc. of blood. Concentration of thymol 0.001 gm. per 1 cc. of blood. Non-protein nitrogen and urea values are uncorrected for ammonia content.

The data presented in Table IV were from a case of severe diabetes under treatment with insulin. Each value shown in the table was checked by a duplicate analysis. The blood was hemolyzed in 5 to 8 days in the incubator at 37–39°C. Even after 33 and 60 days there was no evidence of putrefaction and the only odor detectable was that due to thymol. Not enough blood was available to determine the other constituents.

In all the data presented in the various tables, no precautions were used to prevent contamination by bacteria or other organisms. Non-sterile containers were used, and in all cases the blood was exposed to the air before the bottle was stoppered and

TABLE IV  
*Severe Diabetes Blood Kept at 37–39°C*  
Mg of glucose per 100 cc of blood

Days	Case 93, J B							
	0	3	5	14	23	30	33	60
Monday	376	386	390	375	392	382	376	390
Tuesday	302	328	315	329	321	318	312	Sample exhausted.
Wednesday	375	381	391	379	387	Sample exhausted		
Saturday	390	392	381	399	383	384	Sample exhausted	

Concentration of sodium fluoride 0.01 gm per 1 cc of blood    Concentration of thymol 0.001 gm. per 1 cc of blood.

set aside. In all the determinations for non-protein nitrogen and urea, no corrections were made for the content of ammonia in preserved specimens.

A mixture of 10 parts of sodium fluoride and 1 part of thymol was made. The whole was finely powdered and passed several times through a 100 mesh sieve. A measured amount of this mixture was added to each bottle. For an ordinary analysis a stock of 5 to 12 cc. vials are prepared, and each vial contains an amount of sodium fluoride and thymol to make a final concentration of 0.01 gm. sodium fluoride and 0.001 gm. of thymol or each cubic centimeter of blood.

## CONCLUSION.

From the data obtained and results presented, it is evident that samples of human blood can be preserved for 5 to 6 days, and the values for non-protein nitrogen, urea, uric acid, creatinine, creatine, and sugar will have the same clinical significance as those values obtained by an immediate analysis of the blood. The urea, uric acid, creatinine, creatine, and sugar content of blood can be kept constant for at least 2 weeks. The increase in non-protein nitrogen value cannot be accounted for by an increase in ammonia and amino-acid content.

It is evidently not necessary to draw blood under sterile conditions, except with regard to the patient, in order to preserve blood samples with sodium fluoride and thymol. A mixture of 0.01 gm. of sodium fluoride and 0.001 gm. of thymol for each cubic centimeter of blood is recommended for the preservation of blood for 6 to 14 days.

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## THE PROTEIN MATTER OF BILE.\*

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The protein matter is undoubtedly one of the more important constituents of the bile, but in spite of this fact, only a relatively small amount of work seems to have been done upon it. The small amount of the substance in the fluid, combined with the extreme difficulty of purification, has delayed exact investigation.

The early investigators, Fourcroy, Gmelin, Frommherz, Gugert, Simon, and Berzelius (1), each in turn, made a very superficial study of the properties of bile protein, without much attempt at its purification. Somewhat later, Landwehr (3) claimed that bile protein was a true mucin containing only non-reducing carbohydrates, because he found that boiling it with dilute acids failed to produce any substance which was capable of reducing alkaline copper sulfate. His "mucin" preparation upon analysis gave: C 53.09, H 7.6, O 24.41, N 13.8, and S 1.1 per cent. Later he changed his views and stated that the protein which he analyzed was a mixture of globulin and glycocholic acid. Paijkull (2) showed that if we accept the analysis of Landwehr, the latter's assumption of a mixture of globulin and glycocholic acid was practically an impossibility. Paijkull's own work gave him a product with C 51.67, H 6.88, N 16.09, and S 1.74 per cent. He concluded that bile protein is not a true mucin, although it has some of the properties of a mucin;

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he was inclined to the view that it is a nuclealbumin, but admitted that he was unable to obtain agreement in his phosphorus determinations. Wahlgren (4), Galdi (5), and Cavazzani (6) have recently published individual views regarding the nature of bile protein with some agreement in their conclusions.

It will be seen that the nature of bile protein is still unsettled. Should it be classified as a glucoprotein, a nucleoprotein, or a phosphoprotein; or is it a mixture of two or more of the above? The purpose of this investigation may be stated as (1) the preparation of pure bile protein, followed by its analysis, and (2) the study of its decomposition products and properties in an attempt to classify it properly.

### *Methods and Results.*

Fresh ox bile was filtered through cheese-cloth and used at once to provide against bacterial decomposition of the protein. Many methods of separation and purification were tried, but only the more successful ones will be detailed in this paper. Direct precipitation by adding dilute acid, followed by repeated redissolving and reprecipitation gave a product which still retained pigment. Separation of impurities from acid-precipitated protein by dialysis also proved unsuitable for obtaining a pure protein. Salting out by ammonium sulfate was tried with unsatisfactory results. The following methods gave a product free from bile acids and containing little impurity except traces of bile pigment.

Sodium chloride, with a small amount of HCl, was used to precipitate the protein. The salt and acid brought down a slimy green mass which settled to the bottom, leaving the serum part of the bile almost colorless. The serum was poured off. It was then found that the green mass readily dissolved when stirred with alcohol.

This unexpected result, the solution of the protein in alcohol, must be explained on the assumption that it dissolved under the influence of other constituents. The next step was the addition of acetone until precipitation took place. The small amount of HCl still present probably helped in this last precipitation. The protein was thus obtained as a somewhat green flocculent

precipitate; the greater part of the pigment and a large part of the bile acids were left in solution. Some more of the pigment was removed by extraction with acetone in a Soxhlet apparatus, but after a short time the acetone extraction seemed to reach its limit with color still present. The material was then removed from the Soxhlet apparatus and washed with distilled water until free from chlorides, then after washing repeatedly with alcohol, and finally with ether, the protein was dried for several days at about 40°. A trial Kjeldahl determination upon this material gave only 10.8 per cent N. This pointed to a considerable admixture of bile acids which were removed as follows 300 cc. of distilled water and 5 cc. of 10 per cent NaOH were added to 5 gm. of the dry material. After agitation, complete solution was effected. 600 cc. of 95 per cent alcohol were stirred in and no precipitation took place. 1,500 cc. of acetone and a few drops of dilute HCl brought down a flocculent precipitate of protein. Most of the liquid was poured off and separation was completed by the centrifuge. The protein precipitate was well triturated with alcohol to remove traces of bile acid. After separating once more with the centrifuge, the material was washed with distilled water. It was washed several times with water, then with alcohol and finally with ether, and dried at about 40°. It will be referred to as protein No. 1 a.

Upon being dried the product became somewhat greenish in color, but was free from bile acids. It was insoluble in water and soluble in alkalies. It gave all the protein color reactions more or less distinctly.

In the Kjeldahl determination.

0 25 gm : 0 03387 gm. N = 13 55 per cent N.

0 25 " 0 03441 " " = 13 76 " " "

In the ash determination.

0 2 gm.: 0 0036 gm. = 1.8 per cent ash.

Attempts were made to remove the green pigment from some protein No. 1 a by direct extraction. All the well known organic solvents and some special solvents for bile pigment, provided they were such as could be used under the circumstances were tried, with little success.

In order to obtain a product with less ash, a quantity of bile protein, separated by the NaCl-HCl method, was freed from

bile acids and pigment as far as possible as in the preparation of protein No. 1a, but in addition to this, it was subjected to successive washings with 0.002 N HCl. This was followed by washings with 0.001 N HCl and then several washings with distilled water to free from chlorides. In each washing, separation was effected by the centrifuge. If the isoelectric point of the protein was slightly on the acid side of neutrality, as its solubilities would lead one to expect, such treatment should free the material from metallic ions in accordance with the theory of Loeb (7), and the experience of Van Slyke and Baker (8) and Field (9). The resulting product was greenish in color, but free from bile acids. It will be referred to as protein No. 1 b.

An ash determination gave 1.5 per cent. Some calcium was removed by the acid dialysis. The ash contained some phosphates, and traces of iron were detected.

In the Kjeldahl determination.

0 25 gm.: 0.03367 gm. N = 13.47 per cent N.

After preliminary trials, in which the ordinary methods for determining phosphorus were tried, it was found that a micro method was essential. The method of Wolf and Österberg (10), in a modified form, was finally adopted. This is a combination of Benedict's (11) well known sulfur method, and that of Neumann (12) for phosphorus. The micro method of Raper (13) was substituted for the method of Neumann in my work. The method thus modified was rather laborious, but had an advantage in its economy in material required, since the same sample served for both S and P determinations. In the determination of phosphorus, the produce weighed is lead molybdate, from which the phosphorus is calculated by multiplying by the factor 0.00703.

1 gm protein: 0 0874 gm. BaSO<sub>4</sub>

Control with reagents: 0 0030 " "

0 0844 " = 0 0116 gm. S = 1 16 per cent S.

1 gm protein: 0 1080 " lead molybdate.

0 1080 × 0 00703 = 0 000759 gm. P = 0 075 per cent P.

In later analyses of the same protein by the Raper method direct, omitting the sulfur determination,

0 25 gm. protein: 0 0001767 gm P = 0 070 per cent P.

0 25 " " 0 0001879 " " = 0 075 " " "

The average for these three determinations gave 0.073 per cent P.

In the combustion for C and H.

0.15 gm. protein:	0.2805 gm. CO <sub>2</sub>	and	0.0925 gm. H <sub>2</sub> O.
0 15 "	"	0.2824 "	" " 0 0964 "
0 15 "	"	0.2771 "	" " 0 0956 "
0 15 "	"	0.2765 "	" " 0 0926 "
Average . . . . .	0.2791 "	" " "	0.0942 "
= 50.75 per cent C and 7.02 per cent H.			

The results of the analyses of protein No. 1 b may be summarized as follows: C 50.75, H 7.02, N 13.47, S 1.16, P 0.07, and ash 1.50 per cent.

After completion of analysis of protein No. 1 b, it was determined to try to obtain a product comparatively free from pigments as well as bile acids.

It had been noticed that some chloroform, which had been added as a preservative to a suspension of partially purified protein, had become highly colored. This suggested chloroform as an extractor for the pigment. It had already been tried on dried material without result. Upon experiment it was found that chloroform would dissolve out the pigment best in a slightly acid medium. Efforts were made to ascertain the exact acidity at which the color would leave the protein and pass to the chloroform upon shaking, but it was soon found that the acidity was not the only factor involved. When the protein was precipitated by acid alone, the chloroform failed to extract the pigment. On the other hand, when it was precipitated by acid alcohol, most of the pigment was more readily extracted.

A quantity of ox bile was mixed with an equal volume of 95 per cent alcohol which had been acidified with sulfuric acid. The protein so precipitated was immediately separated, to avoid denaturation by the alcohol. It was then dissolved in water and repeatedly shaken with fresh lots of chloroform and small amounts of acid. Most of the pigment passed into the chloroform. The resulting protein, which had gradually become less soluble in water, was dissolved in dilute sodium hydroxide, salted out by sodium sulfate, and dialyzed against distilled water. It was then washed with alcohol and after extraction with alcohol in

the Soxhlet apparatus, washed with ether and dried. The resulting material was still somewhat stained with pigment. Upon analysis of this protein No. 2,

0 12 gm protein:	0 2080 gm	CO <sub>2</sub>	and 0 0762 gm.	H <sub>2</sub> O.
0 12 " "	0 2110 " "	" "	0 0795 " "	" "
0 12 " "	0 2105 " "	" "	0 0787 " "	" "
Average	0 2098 " "	" "	0 0781 " "	" "
= 47 68 per cent C and 7 23 per cent H.				

In the ash determination.

0 12 gm. protein: 0 0025 gm. = 2 per cent ash

Up to this time, extraction of pigment with chloroform after initial precipitation by acidified alcohol, had given the most promising results in removal of pigment. Further investigation along this line seemed warranted. The fact that protein that had been precipitated by alcohol would give up pigment to chloroform in acid solution, whereas protein precipitated by acid refused to do so, seemed to indicate that in some manner the alcohol had been assisting the chloroform in the extraction in the former experiment with protein No. 2.

95 per cent alcohol and chloroform mixed together were added to bile and the test-tube was shaken. After the separation into the chloroform-alcohol phase below, and the water-alcohol phase above, it was found that very little pigment had left the water-alcohol phase. However, when a little acid was added, and the shaking was repeated, upon separation of the layers, nearly all the pigment was in the chloroform-alcohol below. The water-alcohol phase above was only slightly greenish. The protein was floating as a precipitate at the top of the heavier chloroform layer. It was found that a mixture of 1 volume of chloroform with 3 volumes of 95 per cent alcohol gave good results. To 40 cc. of this mixture, 1 cc. of 25 per cent H<sub>2</sub>SO<sub>4</sub> was added and this was mixed with an equal volume of bile. A precipitate of protein and some bile salts settled on the top surface of the dark colored chloroform layer. The whole was transferred to a separatory funnel and after a few moments, the chloroform, highly colored with green pigment, was drawn off. Since it carried some of the alcohol with it in solution, the next addition of chloroform contained some alcohol to maintain the proportion suitable for

further extraction of the pigment. It was also desirable to keep the bile acids in solution as much as possible, and the extra alcohol tended towards this end. The extraction of the precipitate was repeated in this manner as long as color continued to pass into the chloroform layer. The result was an almost pure white protein somewhat contaminated with bile acids, but giving protein tests well.

The whole experiment was repeated, using 500 cc. of bile with 375 cc. of alcohol, 125 cc. of chloroform, and 13 cc. of  $\text{H}_2\text{SO}_4$ . As before, the alcohol, chloroform, and acid were mixed first and then added to the bile. The temperature rise was not sufficient to promote greatly denaturation of the protein. After repeated treatment with more chloroform and alcohol till pigment was no longer extracted, and the protein product was almost pure white, the chloroform was removed and alcohol added. The material was washed several times with alcohol, then with ether. The ether washings continued to show a slight amount of color, so the material was extracted with ether for several days in a Soxhlet apparatus. It was then dried and found to be free from bile pigment. It was a grayish white powder, by far the best product obtained. However, upon an ash determination, it was found highly contaminated with calcium sulfate, the calcium of the bile having been carried right through the purification process in the form of its sulfate (from the sulfuric acid added). The continued use of alcohol had favored this, as it is well known that calcium sulfate is particularly insoluble in alcohol. (For this reason, in subsequent preparations by the alcohol-chloroform method, it was decided to use hydrochloric acid in place of sulfuric.) The calcium was removed by dialysis in collodion bags against distilled water in a continuous dialyzing apparatus for a considerable time. The protein was then removed from the collodion bags, centrifuged, and washed repeatedly with portions of alcohol. It was then washed with ether and dried at a low heat, about  $30-35^\circ$ , for several days. It will be referred to as protein No. 3 a. Upon analysis,

$$\begin{array}{rcllcl}
 0.12 \text{ gm} & : & 0.2018 \text{ gm} & \text{CO}_2 & \text{and} & 0.0733 \text{ gm} & \text{H}_2\text{O}. \\
 0.12 \text{ " } & & 0.2042 \text{ " } & \text{" } & \text{" } & 0.0782 \text{ " } & \text{" } \\
 \text{Average.} & & 0.2030 \text{ " } & \text{" } & \text{" } & 0.0758 \text{ " } & \text{" } \\
 & & = 46.1 \text{ per cent C} & & & 7.0 \text{ per cent H.} & 
 \end{array}$$

There was too little material for the analysis for other elements.



Another protein preparation had been carried on simultaneously with a slight modification of the above method in that the proportion of the alcohol-chloroform mixture was somewhat increased.

14 cc. of chloroform were mixed with 36 cc. of 95 per cent alcohol and 1 cc. of 25 per cent sulfuric acid was added, giving about 50 cc. in all. With this, 25 cc. of bile were mixed. 8 liters of bile were treated, using these proportions. The remainder of the process was essentially the same as in the preparation of No. 3 a.

After prolonged extraction of the product with alcohol in the Soxhlet apparatus it was washed with ether and air-dried. It was then dried *in vacuo* over sulfuric acid for 10 days at about 35°C. The final product (No. 3 b) was a grayish white powder, slightly hygroscopic, but nearly insoluble in water; insoluble in dilute acids; but soluble in stronger acid. It only partially dissolved in strong acetic acid. It was partially soluble in dilute alkali, forming an opalescent solution. The solution in dilute alkali showed little or no evidence of the mucilaginous consistency which is so characteristic of the native mucins and of the bile itself. This protein, No. 3 b, represents, I believe, the purest product obtained; it was therefore the most valuable for elementary analysis.

In the ash determinations.

0 1	gm	protein:	0 0011	gm	=	1 1	per cent.
0 1	"	"	0 0010	"	=	1 0	" "
0 096	"	"	0 0012	"	=	1 2	" "
Average.....						1 1	" "

In the combustions.

0 1	gm.	protein:	0 1664	gm.	CO <sub>2</sub>	and	0 0693	gm.	H <sub>2</sub> O.
0 1	"	"	0 1625	"	"	"	0 0691	"	"
Average		...	0 1645	"	"	"	0 0692	"	"
= 44 85 per cent C and 7.70 per cent H.									

The figures seemed unexpectedly low for C and too high for H, so a moisture determination was carried out. 0.1 gm. was dried at 90–100° and, after cooling in a sulfuric acid desiccator, it was weighed. Its dry weight was 0.096 gm., a loss of 4 mg. It gained weight rapidly on exposure to the air and soon reached

its former weight. The hygroscopic nature of the material had never, up to this time, been so evident.

Deducting the 4 mg. of moisture in the above combustions on protein No. 3b,

0 096 gm. protein:	0.1664 gm. CO <sub>2</sub>	and	0 0653 gm. H <sub>2</sub> O.
0 096 " " "	0 1625 " " "	"	0 0651 " " "
Average . . . . .	0 1645 " " "	"	0 0652 " " "
= 46.72 per cent C and 7.24 per cent H.			

In the Kjeldahl determination.

0 12 gm. protein:	0 016548 gm. N.
0 12 " " "	0.016520 " " "
Average . . . .	0 016534 " " "
= 13.78 per cent N.	

In the Wolf and Österberg method for S and P.

0 25 gm. protein:	0 0288 gm. BaSO <sub>4</sub> .
0 25 " " "	0 0276 " " "
Average . . . . .	0 0282 " " "
1. Control with reagents:	0 0076 gm. BaSO <sub>4</sub> .
2. " " " "	0 0082 " " "
3. " " " "	0 0066 " " "
Average . . . . .	0 0075 " " "
0.0207 " " " = 1.12 per cent S.	

0 25 gm protein.	0 0819 gm. PbMoO <sub>4</sub>	= 0 230 per cent P.
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0 25 " " "	0 0780 " " "	= 0 219 " " "
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In another P determination, by the method of Raper direct (omitting the S determination).

0 25 gm. protein	0 0847 gm PbMoO <sub>4</sub>	= 0 238 per cent P.
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Average . . . . .	0 23 " " "
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Controls were unsatisfactory, but there was a trace of P in reagents.

The analysis of protein No. 3b may now be summarized as follows: C 46.72, H 7.24, N 13.78, S 1.12, P 0.23, and ash 1.10 per cent.

As will be shown later in this paper, it seems probable that the bile contains at least small amounts of a second protein. When one considers the prolonged treatment necessary for the isolation of the pure protein material, and the difference in the methods, it is quite likely that the final mixtures as analyzed may not contain the same proportion of the two original proteins as they occur in the bile. This would account for the difference in C content. The presence of traces of pigment in No. 1 b would also promote a slight tendency towards a higher C analysis.

The refractive index of alkali solutions of the protein was studied, using an Abbé refractometer and the monochromatic light of the sodium flame. According to an extensive investigation of the refractive index of various proteins by Robertson (14), the addition of relatively small amounts of a protein to a dilute acid, base, or other solvent, changes the refractive index of the solvent.

In the work with this denatured bile protein, 0.02 *N* NaOH was used as the solvent. The temperature was about 25°C. As already stated, the protein was only partially soluble in dilute alkali. Mixtures of different proportions of alkali and protein were made up, shaken, and allowed to stand for some time in order to dissolve all the soluble part. The insoluble portion settled out and the opalescent upper layer was used in taking the readings. No attempt was made to ascertain the exact amount of protein in solution.

No	Protein-alkali mixture	Mean refractive index
1	Control, 0.02 <i>N</i> NaOH.	1.3332
2	0.25 per cent protein.	1.3337
3	0.50 " " "	1.3341
4	0.75 " " "	1.3345
5	1.00 " " "	1.3349

The proteins so far prepared, Nos. 1 a, 1 b, 2, 3 a, and 3 b, were all purified by a more or less prolonged treatment with alcohol. Therefore, they were all denatured to some extent. It seemed desirable to prepare the bile protein in an unchanged form. While the denatured protein served for purposes of analysis, its insolubility in most solvents rendered it of little use for the study of its physical properties. According to the best authorities, denaturation is a form of dehydration. It is a distinct chemical change with a very high temperature coefficient. Chick and Martin (15) found that in the presence of an excess of water, the process of denaturing follows the laws of a monomolecular reaction provided the hydrogen ion concentration is kept constant. According to the investigations of Hofmeister, Bovie (16), and others, and more recently, Young (17), at least three things should be avoided in the preparation of an undenatured

protein; *viz.*, (1) high temperature, (2) the chemical rays of sunlight, and (3) prolonged contact with dilute acids or bases, alcohol or acetone.

In the following method a low concentration of slightly acidified alcohol was used with chloroform as an extractor of bile pigment. The precipitated protein was separated from the alcohol as quickly as possible. Prolonged dialysis against cold distilled water was relied upon to complete the process of purification.

25 cc. of chloroform were mixed with 25 cc. of 95 per cent alcohol and 0.3 cc. of concentrated HCl was added. The whole was mixed with 50 cc. of bile in a separatory funnel. This caused a precipitation of at least most of the protein and gave a good separation of pigment. After immediate removal of the chloroform layer, the protein was again shaken with 10 cc. of fresh chloroform. This was repeated with another 10 cc. of the same solvent. Most, but not all the color was thus extracted, giving a fairly white precipitate of protein with some bile acids. Since the experiment looked promising, the remaining 950 cc. of 1 liter of ox bile were treated in a similar manner with corresponding proportions of alcohol, chloroform, and hydrochloric acid. The product was highly contaminated with bile acids. In previous work, diffusion of these acids through parchment had proved extremely slow. However, Paijkull (2) claims to have succeeded in practically eliminating bile acids in this way. In the writer's work, one portion was dialyzed in the continuous dialyzing apparatus for several weeks during laboratory hours. The flask containing the protein in collodion bags was surrounded with colored paper as a protection against the chemical effect of sunlight. The other portion of protein was dialyzed in collodion bags for about 2 months against distilled water in a dark closet. In both portions, a product was obtained nearly free from bile acids (according to the Pettenkofer test), but some pigment still persisted. No analysis of this protein (No. 4) was undertaken. It was, perhaps, somewhat denatured from the long treatment, but apparently much less so than the former preparation because it was more soluble in dilute NaOH and almost completely soluble in excess of acetic acid. However, it was practically insoluble in water and was precipitated from

alkaline solution upon neutralizing or making slightly acid. It gave positive xanthoproteic and Millon's tests, and responded slowly to the biuret reaction.

According to Hammarsten (18) mucins and nucleoproteins are insoluble in water, except in the form of their alkali salts; *i.e.*, in the presence of traces of alkali. If this be the case, the insolubility in water of the last product is not surprising. In order to explain the solution of the native protein in the bile, we may assume that like the bile acids, it exists in solution in the form of the calcium or sodium salt. However, it is quite possible that it is not in true solution at all. The protein and the bile salts may have a mutual effect upon one another in maintaining their natural dissolved condition. Freezing point determinations, with concurrent analyses of the bile, might throw some light upon this subject, but very little work along this line has been done. Von Rzentkowski (19), working with human bile, found that the freezing point was about  $-0.543^{\circ}$ . This rather slight depression of the freezing point is difficult to reconcile with the comparatively large proportion of salts (organic and inorganic) which are found in the bile. Hence one is inclined to believe that the rather large proportion of organic bile salts (sodium glycocholate and sodium taurocholate) is not producing its maximum effect upon the freezing point. This may best be explained by the assumption that a portion of the bile salts is not in true solution, but is adsorbed upon the protein.

Beside the hypothesis that the material is a nucleoprotein, there are two other alternatives. The phosphorus of the analyses may all be due to the inorganic or organic impurities, in which case the protein must be classed as a glucoprotein; or, the material may be a phosphoprotein (nucleoalbumin) with only a low percentage of phosphorus.

The following experiments were carried out to ascertain the nature of the protein of ox bile:

1. Some protein was kept in 1 per cent NaOH for 24 hours; no phosphorus test with ammonium molybdate was obtained. According to Plimmer and Scott (20), this indicates that it is not a phosphoprotein.

The solution of the problem seemed to hang upon a study of the products of hydrolysis. According to the work of Müller

(21) and his pupils, the true mucins contain a polysaccharide which is easily broken down by boiling with dilute acids. A reducing body is formed, which is glucosamine or a related amino sugar.

2. About 3 gm. of fairly well depigmented protein (which had been precipitated by alcohol) were heated for 20 hours on a boiling water bath with 30 cc. of 10 per cent HCl. A reflux condenser kept the volume constant. A large part of the protein failed to dissolve, but remained in the form of dark sticky lumps. The product was filtered and the filtrate showed no reducing action upon alkaline copper sulfate. Similar experiments with 2.5 per cent HCl, concentrated HCl, and 10 per cent HCl and dilute  $H_2SO_4$  resulted in failure to produce a reducing sugar.

It is evident from the results of the above experiments that bile protein either does not contain a reducing sugar of the glucosamine type, or if such a body is present, it is very difficult to release by the usual procedure of acid hydrolysis. It should be noted here that Paijkull (2) had a similar experience in his attempts to obtain a reducing substance from bile protein; Landwehr (3), as stated earlier in this article, explained this lack of reducing power in the products of hydrolysis by assuming that the carbohydrate in bile protein was of such a character that it could not furnish any reducing substance.

Bile protein treated with dimethylaminobenzaldehyde after the manner of Ehrlich gave a rose color, a reaction said to be positive with all glucoproteins.

Some further hydrolysis experiments were then carried out. Levene and La Forge (22), in their work on the mucoids, brought about incipient hydrolysis by allowing the protein to stand for some hours in dilute alkali; *e.g.*, 2 per cent KOH or lime-water. Some partially purified bile protein was placed in 2 per cent NaOH at about 40°C. for a short time and then left at room temperature for about a week. The product was then neutralized with HCl and excess acid was added to make about a 10 per cent solution of HCl. After boiling about 3 hours the solution was found to have slight reducing power by the usual test.

It would seem from this last experiment that the dilute alkali had some hydrolyzing effect that acid treatment alone could not produce. The fact that the reducing power was so small may

be interpreted in two ways: (a) If the bile protein were a chemical individual and all its molecules capable of furnishing a reducing body, then only a few of them were attacked by the alkali. (b) On the other hand, this slight reducing power might come from the hydrolysis of a second protein which represented only a small part of the total protein present.

During the hydrolysis experiments, tests were made for the presence of purine bases in the various solutions. The ammoniacal silver nitrate test appeared to be slightly positive after standing several hours, but the amount of the brown precipitate was too small for confirmation. The same precipitate was again formed upon repeating the test with some other material. Other purine tests gave negative results.

Pyrimidine bases were not present in the free state as judged by qualitative tests. However, since they are more difficult to split off from the nucleic acid than the purine bases this is not surprising.

The evidence points to the presence of a small admixture of nucleoprotein with the main body of protein as separated from the bile.

Some purified bile protein was placed in a 30 per cent nitric acid solution and kept at about 35°C. for several days. The molybdic acid-phenylhydrazine test for phosphates was then applied. No separation of phosphates as a result of the nitric acid treatment could be detected. According to Macallum (23), nucleoproteins split off phosphorus under such treatment.

#### CONCLUSIONS.

The positive Molisch and Ehrlich reactions and the N content of 13.5 to 13.8 per cent point to the glucoprotein molecule, nucleoproteins usually show from 15 to 17 per cent N. The very low P content (0.07 to 0.23 per cent) as shown by analysis and the negative result of the test for P after the action of 30 per cent  $\text{HNO}_3$  seem to exclude the possibility of more than traces of nucleoprotein being present. The negative result of the Plimmer test with 1 per cent NaOH and the low P content certainly do not indicate that the material contains any large proportion of a phosphoprotein. The apparently faintly positive test for purines is evidence for the presence of traces of nucleo-

protein; the difference in C content of products which had been precipitated and purified by widely different procedures can best be interpreted by the assumption that a mixture of proteins is found in ox bile. The solubilities of the material furnish no clue whatever, and the lack of any considerable amount of reducing carbohydrate in the hydrolyzed material remains unexplained. Nevertheless, the bulk of the evidence seems to point to a mixture of a large proportion of some very stable form of glucoprotein with a much smaller amount of nucleoprotein.

#### SUMMARY.

1. The protein of ox bile has been isolated and purified according to several different methods; many other procedures along this line have been extensively investigated.

2. Three of the above methods gave a product which was undoubtedly denatured; the fourth we believe to be unchanged, or at least very slightly denatured.

3. Elemental analyses have been carried out upon two of these purified products for O, H, N, S, and P. C and H have also been determined with other products obtained by more or less modified procedures.

4. A study of the reactions and of the products of hydrolysis of ox bile protein has been made with a view to establishing the nature of the substance.

5. Evidence has been gathered which tends to show that the protein of ox bile is a mixture of a comparatively large proportion of glucoprotein with a small amount of nucleoprotein.

In conclusion, I desire to express my thanks to Prof. A. B. Macallum for many valuable suggestions in connection with the carrying on of this work.

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# THE NUTRITIONAL REQUIREMENTS OF BABY CHICKS.

## III. THE RELATION OF LIGHT TO THE GROWTH OF THE CHICKEN.\*

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PLATES 1 AND 2.

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The therapeutic action of light has been studied experimentally, and through the work of Huldshinsky, Hess, Powers, and others (1) its relation to the prevention and cure of rickets has been established. Further, it has been shown through a number of investigations that the light from various sources can maintain calcium and phosphorus equilibrium in the blood stream (2).

Properties similar to those of radiant energy in respect to the functions mentioned above are possessed by green plant tissue (3), fish liver oils, and so far as known in a limited degree by certain foodstuffs, as for example the yolk of the egg (4). To this factor in foodstuffs is assigned the term antirachitic vitamin. Its distinction from vitamin A was made probable by the work of McCollum, Simmonds, Becker, and Shipley (5), and further confirmed by the work of Steenbock and Nelson (6) and Steenbock, Hart, Jones, and Black (7). Our knowledge of its distribution in nature is still limited.

In the presence of all known dietary factors, but in the absence of the antirachitic vitamin or its equivalent not only will imper-

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fect skeleton formation result and abnormal calcium to phosphorus ratios exist in the blood, but growth will cease. Suggestion of the equivalence of fat-soluble vitamins to light and their relation to growth has been made by Hume (8), but with the incorrect assumption that it was vitamin A that functioned as the equivalent. Goldblatt and Soames (9) have also observed the relation of the light from a quartz mercury vapor lamp to growth. Powers, Park, and Simmonds (10) recognized the equivalence of light from various sources to the antirachitic properties of cod liver oil as distinct from vitamin A and the development of a condition of physical vigor in the animals so exposed, but did not stress the relation of light to growth. However, in a recent paper by Steenbock and Nelson (6) where control of the experimental animals in respect to vitamin storage and light was adequately recognized, the close relation between the antirachitic vitamin (or its equivalent in light) and growth was given sound experimental support.

The fact that the baby chick is very susceptible to rickets (11) and that this pathological condition can be prevented by the use of cod liver oil led us to investigate the problem of the relation of light to the growth of this species. In nature the young hatch of early spring follows the hen in search of food with a variable intake of insects and green plant tissue. Leg weakness or rickets of chicks reared under such conditions is unknown to us. Leg weakness is a malady of early spring hatching, coupled with confinement and ignorance of proper dietary relations. The commercial poultryman with his early spring hatch uses freshly sprouted grains or stored succulent plant tissue such as roots or cabbage as supplements to his ration with the hope of avoiding nutritional trouble. He anxiously awaits the advent of warm weather when he can get the bird out of doors on freshly turned ground—believing that the provision of a “scratch” is necessary for success. We now know that the chick can be carried to maturity under strict confinement (11) provided the proper dietary regimen is furnished.

It is apparent from all our data that the chick requires a liberal supply of the antirachitic vitamin or its equivalent. The recognition of light as the equivalent or supplement to the antirachitic factor made it seem very probable to us that light could play an important part in the practical rearing of this species.

## EXPERIMENTAL.

In our preliminary studies of this problem of the relation of light to the growth of the baby chick, we have chosen as our ration one which will invariably lead when used under housed conditions to cessation of growth, symptoms of rickets or leg weakness, although often the bird dies suddenly without manifesting severe rachitic symptoms. The basal ration was composed of 97 parts of white corn, 2 parts of calcium carbonate, 1 part of common salt, and separator skimmed milk *ad libitum*. This ration is not abundant in vitamin A although some is present. From our records with chicks it also appears to be low in the antirachitic vitamin.

In the experiments to be reported in this paper groups of 8 pure-bred White Leghorns were taken at hatching. They had been hatched from eggs laid by hens which had all received similar rations, thereby minimizing variations in the storage of nutritive factors in the egg. Group I, designated as "no light group," was placed in the basement of the University poultry building where there was no direct sunlight—the room being dimly lighted through closed basement windows. The chicks were allowed a runway with a shavings litter. Granite grits were allowed all groups. Group II, "full sunlight group," was placed out of doors in a small covered coop with a raised runway of boards screened with wire netting, allowing all day access to sunlight, but preventing access to extraneous food materials. The floor was covered with pine shavings. Groups III, IV, and V were kept in an attic with darkened skylights and without access to direct light. They also were kept on pine shavings. Group III received 10 minutes daily exposure to sunlight. Group IV received 1 hour exposure to sunlight daily. Group V was radiated 10 minutes daily, except Sunday, by the rays of a quartz mercury vapor lamp.

This experiment was started July 18. There were days when the weather was cloudy, but exposures were made on all days except where rain prevented. The records of the weights are given in Tables I to V, inclusive, and Figs. 1 and 2, showing pictures of birds exposed to sunlight for 10 minutes (Group III), and all day (Group II), respectively. All the birds of Group I (no sunlight group) were dead by the time the pictures of these groups were taken.

TABLE I.

*Weight Record of Group I, Fed White Corn, Skimmed Milk, with No Sunlight.*

No.	Initial weight	2 weeks.	4 weeks.	6 weeks.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
4863	38	64	80	X-rayed.
4864	38	64	107	Dead.
4865	38	67	77	"
4866	41	64	92	"
4867	40	66	Dead.	
4868	34	Dead.		
4869	37	58	89	Dead.
4870	37	61	107	"

TABLE II.

*Weight Record of Group II, Fed White Corn, Skimmed Milk, Plus Sunlight All Day.*

No	Initial weight	2 weeks	4 weeks	6 weeks.	8 weeks.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
4881	36	70	128	210	237
4882	36	75	139	210	290
4883	36	55	65	X-rayed.	
4884	33	61	109	"	
4885	35	57	87	175	230
4886	38	71	100	202	261
4887	34	72	Dead.		
4888	37	62	102	179	235
4889	36	Dead.			
4890	35	77	105	Dead.	

TABLE III

*Weight Record of Group III, Fed White Corn, Skimmed Milk, Plus Sunlight 10 Minutes.*

No	Initial weight	2 weeks	4 weeks	6 weeks	8 weeks
	<i>gm</i>	<i>gm</i>	<i>gm.</i>	<i>gm</i>	<i>gm.</i>
5090	37	64	90	Dead.	
5091	36	60	75	"	
5092	38	50	60	"	
5093	37	56	85	X-rayed.	
5094	36	57	85	"	
5095	34	Dead.			
5096	38	51	75	140	205
5097	37	52	90	145	215
5098	36	87	135	180	Dead.
5099	38	72	95	95	"

We do not desire at this time to emphasize the possible quantitative differences in the effect of the kind of light or the length of time of exposure. All we wish to do is to call particular attention to the distinct differences in the growth records of Groups

TABLE IV.

*Weight Record of Group IV, Fed White Corn, Skimmed Milk, Plus Sunlight 1 Hour.*

No	Initial weight	2 weeks	4 weeks	6 weeks	8 weeks.
	gm	gm	gm.	gm	gm.
5100	41	70	140	230	Dead.
5101	37	57	Dead.		
5102	40	Dead.			
5103	42	"			
5104	33	65	Dead.		
5105	33	72	140	175	X-rayed.
5106	36	65	110	X-rayed.	
5107	38	58	80	90	Dead.
5108	40	80	125	225	290
5109	36	68	100	120	Dead.

TABLE V

*Weight Record of Group V, Fed White Corn, Skimmed Milk, Plus 10 Minutes Daily Radiation (Ultra-Violet).*

No	Initial weight.	2 weeks.	4 weeks	6 weeks.	8 weeks
	gm	gm.	gm	gm	gm
5110	41	85	145	260	
5111	35	Dead.			
5112	37	77	Dead.		
5113	32	60	75	Dead.	
5114	36	62	90	95	Dead.
5115	38	69	110	X-rayed.	
5116	35	72	115	165	X-rayed.
5117	37	74	115	160	Dead.
5118	38	87	135	250	255
5119	38	68	Dead.		

I and II—the group receiving no sunlight and the group receiving sunlight the entire day. Those receiving no sunlight terminated their existence in 6 weeks, reaching weights of approximately 100 gm. These birds were listless and inactive, rough of feather,

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straddling, and awkward in gait, squatting frequently. Death often occurred suddenly. Possibly their nutritional failure was partly due to a low supply of vitamin A in the ration used. However, no distinct ophthalmias were observed.

The group reared in the "all day sunlight" exhibited signs of behavior the exact antithesis of the birds of Group I. They were alert and active with no hesitation in walking, no frequent squatting, and the feathers of most of these birds were relatively smooth, although in this regard they were not perfectly normal. The distinction between the two groups was very remarkable and as far as can be discerned at present, the only difference between the rations allowed was direct sunlight acting as the equivalent or supplement to a limited supply of the antirachitic vitamin. In 8 weeks five of these birds had reached weights averaging 250 gm.

In respect to the other groups, all we can say at present is that the group receiving sunlight 10 minutes daily (Group III) was more like Group I (no light group) in general behavior, while those receiving sunlight for 1 hour daily (Group IV) and 10 minutes of ultra-violet light (Group V) grew better and lasted longer than those of Groups I and III. However, they were not equal as a group to the "all day sunlight" group (Group II).

While we recognize the occurrences of the antirachitic factor in green plant tissue (3) and the common use by poultrymen of greens, yet no adequate and complete data are yet available on the quantitative relation in respect to the antirachitic properties of sunlight as compared with green plant tissue. We do wish, however, to present the limited data we have available, bearing on this point. In other studies on the rearing of baby chicks we had occasion to use a basal synthetic diet supplemented with carefully dried green clover and also supplemented with *fresh* green clover equivalent in dry matter to 5 per cent of the ration. The basal ration consisted of 18 per cent of alcohol-extracted casein, 60 per cent of dextrin, 5 per cent of salt mixture 32, 2 per cent of agar, and 15 per cent of dried brewer's yeast. The green clover in proper quantity was chopped up daily and mixed with the ration. A group of eight Barred Rock chicks were involved in this experiment. They were from the same hatch and the eggs were from hens of similar nutritional history. These birds

were kept out of direct sunlight in our attic and provided with a runway covered with shavings. They were started on this ration June 19, immediately after hatching. On July 12 or after a lapse of 23 days and with good consumption of the ration it became apparent that the provision of this amount of green food in the ration would not allow normal growth or even maintenance. Consequently, two chicks from this group (Nos. 7 and 8) were given in addition to their ration plus green food a daily exposure of  $\frac{1}{2}$  hour to sunlight. The remainder of the group were allowed to terminate their course without change of environment. For records of this group see Table VI. They were all dead or

TABLE VI

*Record Weight of Chicks Fed Basal Synthetic Ration Plus Green Clover, Equivalent to 5 Per Cent of the Dry Matter of the Ration, and Kept Out of Direct Light Nos. 7 and 8 Received the Same Ration but When 23 Days Old Received in Addition  $\frac{1}{2}$  Hour of Exposure to Sunlight Daily.*

No	Initial weight	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
1	35	57	72	80	Dead	
2	36	53	Dead.			
3	35	62	87	95	95	Dead X-rayed and photographed.
4	33	60	Dead			
5	31	60	55	Dead.		
6	31	60	77	"		
7	32	58	85	145	260	410
8	38	77	105	180	305	X-rayed and photographed.

stationary in weight in from 6 to 8 weeks. The two birds, exposed to sunlight, made continued and excellent growth. No. 8 at 8 weeks of age had reached the weight of 305 gm. when it was photographed and radiographed for purpose of contrast with No. 3 a bird from this group that was unexposed to light and which at 8 weeks of age weighed 95 gm. At this time No. 3 was also photographed and radiographed (see photographs and radiographs, Figs. 3 and 4). No. 7 is still growing and at this writing weighs 520 gm., and appears to be a normal specimen. Apparently the chick requires a liberal amount of the antirachitic vitamin or its



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equivalent, and the amount of green material we furnished was insufficient to meet its needs unless sunlight in addition was provided.

### SUMMARY.

It is apparent from our data that light can play a very important part in the rearing of baby chicks, acting as a supplement or the equivalent to the antirachitic factor of foodstuffs.<sup>1</sup>

From limited data it appears that  $\frac{1}{2}$  hour daily exposure to direct sunlight was much more potent in furnishing the antirachitic equivalent than was 5 per cent of a synthetic ration fed as fresh green clover, calculated on the basis of the dry weight of the clover.

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<sup>1</sup>Data in agreement with our conclusions were presented by Dr. J. S. Hughes, Kansas State Agricultural College, at the September, 1923, meeting of the American Chemical Society, Milwaukee, Wisconsin

## EXPLANATION OF PLATES.

## PLATE 1.

FIG. 1. The effect of limited radiant energy (sunlight) and a rachitic ration. Fed white corn 97, calcium carbonate 2, sodium chloride 1, plus skimmed milk *ad libitum*. Sunlight 10 minutes per day. Weight 140 gm. at 6 weeks of age. An abnormal specimen.

FIG. 2. The effect of unlimited radiant energy (sunlight) and a rachitic ration. Fed white corn 97, calcium carbonate 2, sodium chloride 1, plus skimmed milk *ad libitum*. Exposed to sunlight all day. Weight 210 gm. at 6 weeks of age. Apparently a normal specimen.

FIG. 3. A contrast between the effect of green plant tissue and radiant energy. Both chicks received a synthetic ration composed of alcohol-extracted casein 18, dextrin 60, salt mixture 5, agar 2, yeast 15, plus 5 per cent of fresh green clover, calculated on the basis of the dry weight of the clover.

	No 3 (left)	No 8 (right)
	gm.	gm
Initial weight, June 19, 1923 .. .	35	38
Weight, July 10, 1923	77	95
July 12, 1923, began exposure of No. 8 to sunlight $\frac{1}{2}$ hour daily, both birds being continued on the same ration.		
Weight, Aug. 8, 1923 . . .	95	235

Photographed Aug 9, 1923. (See record of other individuals similarly treated, Table VI )

## PLATE 2.

FIG. 4. A radiograph of Birds 3 and 8 (photograph, Fig. 3). No 3 (left) received the synthetic ration plus green plant tissue, while No. 8 (right) received the same ration plus  $\frac{1}{2}$  hour daily exposure to sunlight. Normal calcification of the skeleton of No 3 had not taken place. The patella and metatarsus are but dimly outlined. In the case of No. 8 the skeleton is sharply outlined and great improvement in calcium and phosphorus deposition must have occurred.



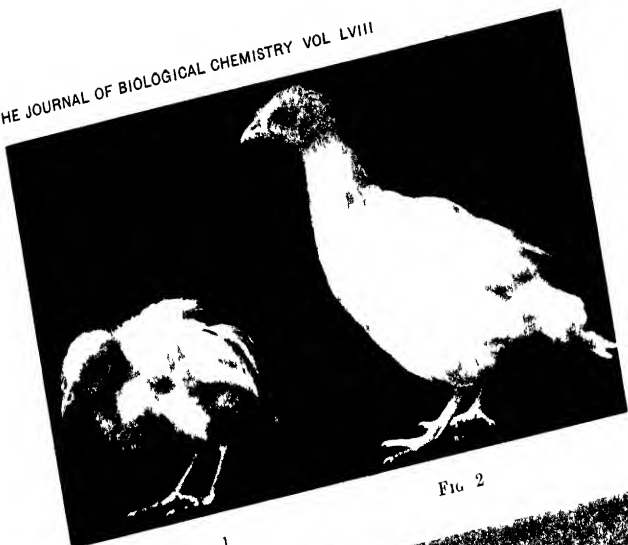


FIG. 1

FIG. 2



FIG. 3.







## **DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.**

### **IV. THE COMPARATIVE EFFICIENCY OF MIXED GREEN GRASSES AND THIS SAME MIXTURE PLUS STEAMED BONE MEAL IN MAINTAINING CALCIUM AND PHOSPHORUS EQUILIBRIUM IN MILKING COWS.\***

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In previous papers (1) from this laboratory dealing with the subject of calcium assimilation by goats and cows, evidence has been presented showing: (1) that green plant tissue contained more of a vitamin assisting calcium assimilation than plant tissue exposed to long curing processes, (2) that alfalfa hay when fed as a supplement to grains and grain by-products may be so cured as to retain quantities of this vitamin sufficient to maintain liberally milking cows in positive calcium and phosphorus balance; but under the practical conditions of curing hay under caps the efficiency of such dried alfalfa was not equivalent in this respect to green alfalfa; (3) that alfalfa hay cured in the windrow with exposure to air and light for 4 days was not capable of maintaining calcium equilibrium in liberally milking cows; and (4) that with timothy hay as the principal roughage for milking cows or the same hay supplemented with bone meal, positive calcium and phosphorus balances could not be established. In other words, while the losses of calcium were somewhat reduced by supple-

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menting the timothy hay ration with a calcium salt, storage of calcium was impossible and even equilibrium was not established.

The accumulation of the above facts briefly summarized from our earlier publications paved the way for further study of a question of considerable practical moment; namely, can mixed green grasses, constituting the common summer pasturage for dairy cattle, provide an ample supply of all the factors necessary to establish conditions for calcium and phosphorus storage when supplemented with grains, grain concentrates, and corn silage, or failing in this, can it be accomplished by supplementing with calcium salts such as rock phosphate or steamed bone meal? These data would also give an answer to the question of the most effective time to establish the calcium and phosphorus reserves of dairy cattle. While in the winter feeding of liberally milking cows, equilibrium of calcium and phosphorus can be maintained by the use of an alfalfa hay cured in a special way and properly supplemented with grains, grain by-products, and corn silage, yet such methods of curing may not be economical and the most effective method of lessening the mineral draft on high milking cows would be through an efficient upbuilding of the calcium and phosphorus reserves on supplemented green grasses. If this is not possible it must then be done during the dry period. In addition, an answer must ultimately be given to the question of the effect of long continued negative calcium and phosphorus balances, even when drawn from built up reserves, upon the fecundity and reproducing potency of dairy cattle (2).

This investigation gives no answer to the last question, but it does give a partial answer to the first question and also discloses a probable fourth factor operating in experimental inquiries into the maintenance of the calcium and phosphorus reserves of liberally milking cows. This fourth factor involves the nutritional history of the animal before its use for experimental purposes, and its possible stored reserves.

#### *Experiment I.*

In the experiment to be described we believe we established ideal conditions for calcium and phosphorus storage through the provision of a ration containing a liberal supply of calcium, phosphorus, and the vitamin assisting in calcium assimilation. This

was done by using a ration made up of grains, corn silage, and fresh green plant tissue composed of June Grass (*Poa pratensis*) and white clover (*Trifolium repens*). Our records, however, indicate that for these liberally milking animals the calcium supply was too low with the unsupplemented ration. On the other hand, the phosphorus supply, mainly provided through the grain portion of the ration, appeared to be ample. In the second period of this experiment the ration was supplemented with steamed bone meal<sup>1</sup>—each animal being allowed 200 gm. per day. All these animals were heavy milkers, yielding approximately 50 lbs. of milk daily.

The ration fed consisted of 40 lbs. daily of fresh green grass, 20 to 25 lbs. of corn silage, and a grain mixture made up of 60 parts of yellow corn, 25 parts of wheat bran, and 15 parts of oil meal. For 3 lbs. of milk produced, 1 lb. of the grain mixture was fed daily. The fresh grasses were cut daily from a lawn on the University campus and 20 lbs. per individual set aside for both evening and morning rations. In addition these grasses were sampled daily for calcium and phosphorus estimations.

The procedure followed in these metabolism experiments was similar to those described in our earlier experiments of like character. Quantitative collection of all excreta and milk was made with fractional sampling of both feces and urine for final analysis for calcium and phosphorus.

In spite of the provision of this supposedly ideal ration, a negative calcium balance was recorded for Animal 1 in the unsupplemented period, but with equilibrium in respect to calcium and phosphorus in the supplemented period. (See records in Tables I, II, and III.) For Animal 2 a negative calcium balance prevailed in both unsupplemented and supplemented periods; the positive balance recorded in the 1st week of the supplemented period is due to a lag in the excretion of calcium and should be disregarded. In the case of Animal 3 a negative calcium balance was observed in the unsupplemented period, but distinctly positive balances for both calcium and phosphorus were recorded in the period supplemented with bone meal.

The varying results with the three animals probably rest upon

<sup>1</sup> This bone meal was a product specially produced by the United Chemical and Organic Products Co., Chicago, Illinois, for feeding purposes.

their previous nutritional history and introduce a fourth factor of considerable moment in an experimental inquiry into the problem of maintaining the calcium and phosphorus reserves of liberally milking cows. This is the tentative explanation we have to offer since the milk production of the three cows was much alike,

TABLE I  
*Record of Calcium Balance and Milk Production of Animal 1*

Period.	CaO in feces	CaO in urine	CaO in milk.	Total CaO excreted	Total CaO intake	Balance per week.	Balance per day.	Milk per week
Green grass period								
	gm	gm	gm.	gm	gm	gm	gm	lbs
May 16-22	551 93	2 08	304 65	858 66	479 59	-379.07	-54 15	364.7
" 22-29	268 70	3 38	278 75	550 83	488 10	-62 73	-8 96	353 9
" 29-June 5	273 15	1 31	285 97	560 43	485 48	-74 95	-10 71	352 9
Green grass + bone meal period								
June 5-12	682 90	7 42	263 47	953 79	1,203 42	+249 63	+35 66	338 4
" 12-19 ..	827 11	6 95	246 77	1,080 83	1,026 65	-54 18	-7.74	312 3
" 19-26...	971.14	9 03	237 24	1,217 41	1,280 94	+63 53	+9 08	312 9

*Record of Phosphorus Balance of Animal 1.*

Period.	P <sub>2</sub> O <sub>5</sub> in feces	P <sub>2</sub> O <sub>5</sub> in urine	P <sub>2</sub> O <sub>5</sub> in milk	Total P <sub>2</sub> O <sub>5</sub> excreted	Total P <sub>2</sub> O <sub>5</sub> intake	Balance per week.	Balance per day
Green grass period.							
	gm	gm	gm	gm	gm	gm.	gm
May 16-22 .	1,125 00	2 76	326 18	1,453 94	1,292 36	-161 58	-23 08
" 22-29 ..	867 69	2 68	323 86	1,194 23	1,301 15	+106 92	+15 27
" 29-June 5	855 36	2 95	330 02	1,188 33	1,313 10	+124 77	+17 82
Green grass + bone meal period.							
June 5-12 .	1,228 10	3 66	322 04	1,553 80	1,832 36	+278.56	+39 79
" 12-19 ..	1,359 07	3 11	302 08	1,664 26	1,554 41	-109 85	-15 69
" 19-26. . .	1,325 20	3 70	294 06	1,622 96	1,741 37	+118 41	+16 91

although, as will be pointed out later, the calcium secretion in the milk of Animal 3 was distinctly lower than that in Animal 2.

The three cows used were pure-bred Holsteins. Nos. 1 and 2 had been milking since January 25 and 4, 1922, respectively, but had received an alfalfa hay ration during this period and had

also been upon green pasture just previous to being used in this metabolism experiment which started May 15, 1922. The use of such rations probably did not result in a serious or extended depletion of the mineral reserves and consequently no pronounced storage was possible even with our ideal ration. In the case of Animal 3 which freshened March 15, 1922, the preexperimental

TABLE II.  
*Record of Calcium Balance and Milk Production of Animal 2.*

Period	CaO in feces	CaO in urine	CaO in milk	Total CaO excreted	Total CaO intake	Balance per week	Balance per day.	Milk per week.
Green grass period.								
May 16-22	gm 461 86	gm 2 57	gm. 322 62	gm. 787.05	gm. 479 59	gm. -307 46	gm. -43 92	lbs. 386 2
" 22-29.	309 32	3 16	323 76	636 24	488 10	-148 14	-21 16	369 5
" 29-								
June 5...	310 37	2 42	285.06	597 85	485 48	-112 37	-16 05	365.1

Green grass + bone meal period.

June 5-12	692 64	5 48	263 60	961 72	1,203 42	+241 70	+34 53	352 0
" 12-19	1,039 16	4 20	266 90	1,310 26	1,158 15	-152 11	-21 73	343 8
" 19-26	1,118 26	8 52	258 81	1,385 59	1,280 94	-104 65	-14 95	347 6

*Record of Phosphorus Balance of Animal 2.*

Period.	P <sub>2</sub> O <sub>5</sub> in feces	P <sub>2</sub> O <sub>5</sub> in urine	P <sub>2</sub> O <sub>5</sub> in milk	Total P <sub>2</sub> O <sub>5</sub> excreted	Total P <sub>2</sub> O <sub>5</sub> intake	Balance per week	Balance per day
Green grass period.							
May 16-22....	gm 933.32	gm 3 36	gm 355 93	gm 1,292 61	gm 1,292 36	gm -0 25	gm -0 04
" 22-29 ....	910 15	2 50	330 47	1,243 12	1,301 15	+58 03	+8 29
" 29-June 5	955 26	2 09	341 41	1,298 76	1,313.10	+14 34	+2 04

Green grass + bone meal period.

June 5-12.. . .	1,354 50	2 34	322 72	1,679 56	1,832.36	+152 80	+21 83
" 12-19....	1,494 53	4 61	326 21	1,825 35	1,714 90	-110 45	-15.78
" 19-26.. . .	1,469 82	5 87	323 51	1,799 20	1,741 37	-57.83	-8 26

ration had been a grain mixture, corn silage, and timothy hay grown on an acid soil and low in calcium. This animal was a liberal milker and such a ration must have led to a distinct mineral depletion before being placed in our experimental inquiry and also to a depletion of the vitamin concerned in calcium assimilation.

These differences in results secured from our experimental ration with the several animals correlate with the differences in the preexperimental feeding period and raise the question whether or not the liberally lactating cow does not draw upon mineral reserves with greater ease than on the food supply, at least up to a certain point, even when the ration is an ideal one for calcium and

TABLE III  
*Record of Calcium Balance and Milk Production of Animal 3.*

Period	CaO in feces	CaO in urine	CaO in milk	Total CaO excreted	Total CaO intake	Balance per week.	Balance per day	Milk per week
Green grass period.								
May 29-June 5	gm. 280 61	gm. 3 19	gm 219 45	gm 503 25	gm 450 71	gm -52 54	gm -7 51	lbs 326 6
Green grass + bone meal period.								
June 5-12 ..	515 05	2 98	218 58	736 61	1,168 65	+432 04	+61 72	325 3
" 12-19 ..	926 40	4 08	192 22	1,122 70	1,130 19	+7 49	+1 07	306 8
" 19-26 . . .	919 63	5 99	222 01	1,147 63	1,253 00	+105 37	+15 05	332 8

*Record of Phosphorus Balance of Animal 3.*

Period	P <sub>2</sub> O <sub>5</sub> in feces	P <sub>2</sub> O <sub>5</sub> in urine	P <sub>2</sub> O <sub>5</sub> in milk	Total P <sub>2</sub> O <sub>5</sub> excreted	Total P <sub>2</sub> O <sub>5</sub> intake	Balance per week	Balance per day
Green grass period.							
May 29-June 5 .	gm 765 88	gm 2 93	gm 305 35	gm 1,074 16	gm 1,188 62	gm +114 46	gm +16 35
Green grass + bone meal period.							
June 5-12. . .	1,237 93	2 58	313 09	1,553 60	1,707 88	+154 28	+22.04
" 12-19 .	1,300 05	2 97	305 05	1,608 07	1,599 28	-8 79	-1 26
" 19-26 . .	1,169 87	3 35	327 72	1,500 94	1,625 75	+124 81	+17 83

phosphorus assimilation and storage. The results also indicate that the degree of mineral and probably vitamin depletion before the period of collection of the experimental data is an important factor in measuring the comparative efficiency of feeding materials for calcium and phosphorus storage. For example, for Animal 2 our experimental ration would be judged inefficient in respect to its power to maintain calcium and phosphorus equilibrium; while in the case of Animal 3 it showed itself highly effective.

Both animals were producing approximately equal quantities of milk, but the percentage of calcium in the milk of Animal 2 was slightly higher than in the milk of Animal 3. In the case of No. 2 the percentage of calcium oxide in the milk was approximately 0.167. In the case of No. 3 it was 0.144. In the supplemented period Animal 2 was secreting approximately 260 gm. of calcium oxide per week in the milk; while Animal 3 was secreting approximately 210 gm. in the same time with the intake of Animal 2 slightly higher than that of Animal 3. It is entirely possible that individual differences in respect to the efficiency of calcium assimilation can exist, and had the plane of calcium intake been higher in the case of No. 2, we would have established calcium equilibrium. This is suggested as an alternative explanation for the striking differences in the calcium records of Animals 2 and 3.

### *Experiment II.*

Because of the variations in results secured in the first experiment and the difficulty of their interpretation, the experiment was repeated, but using only animals whose previous nutritional history was known, especially in respect to the character and time of use of the roughage part of the ration. Animal 4 (a pure-bred Holstein) had received a good grade of alfalfa hay purchased on the open market, but of unknown curing exposure, in addition to corn silage and a suitable grain mixture. This ration was fed from January 24 to May 14, 1923, the date of initiation of the metabolism experiment. No. 4 freshened December 16, 1922, and was bred April 23, 1923. We had no guarantee that this animal, milking 50 lbs. per day, would not be in negative calcium balance in the preexperimental period, but we believed that through the use of this alfalfa hay pronounced depletion of calcium was less likely to occur as compared with the use of a timothy hay or corn stover grown on acid soils. Consequently, Animal 5 (a pure-bred Jersey) was placed on a grain mixture, corn silage, and a timothy hay of poor quality from January 24 to May 14, 1923, the date on which the metabolism experiment started. This animal freshened December 6, 1922 and was bred January 20, 1923. Animal 6 (a grade Holstein) was prepared for the metabolism experiment in a similar manner, placing her on a grain mixture and corn stover grown on acid soil. She received no corn silage and was started

on the above ration in October, 1922, continuing on it until the initiation of the metabolism experiment on May 14, 1923. This animal was fresh October 17, 1922, and bred March 25, 1923. From previous experience we felt sure that Animals 5 and 6 would be in a depleted condition particularly in respect to calcium

TABLE IV.

*Record of Calcium Balance and Milk Production of Animal 4.*

Period	CaO in feces	CaO in urine	CaO in milk	Total CaO excreted	Total CaO intake	Balance per week.	Balance per day.	Milk per week.
Green grass period.								
May 14-21	420 84	7 06	271.92	699 82	538 84	-160 98	-22 99	334 6
" 21-28	310 02	0 53	271 68	582 23	551 32	-30 91	-4 41	328 8
" 28- June 4	381 24	0 95	267.72	649 91	576 31	-73 60	-10 51	327.6
Green grass + bone meal period								
June 4-11	807 53	2 01	254 74	1,064 28	1,191 71	+127 43	+18 20	310.0
" 11-18	1,001 70	19 90	249 80	1,271 40	1,218 28	-53 12	-7.59	292.6
" 18-24	806 66	3 43	224 00	1,034 09	1,050 70	+16 61	+2 37	246 7

*Record of Phosphorus Balance of Animal 4*

Period	P <sub>2</sub> O <sub>5</sub> in feces	P <sub>2</sub> O <sub>5</sub> in urine	P <sub>2</sub> O <sub>5</sub> in milk	Total P <sub>2</sub> O <sub>5</sub> excreted	Total P <sub>2</sub> O <sub>5</sub> intake	Balance per week.	Balance per day
Green grass period.							
May 14-21..	708 75	6 04	311 41	1,026 20	1,036 51	+10 31	+1 47
" 21-28 . . .	783 64	7 34	301 54	1,092 52	1,063 31	-29 21	-4 17
" 28-June 4	710.01	4 88	300 44	1,015 33	1,144 09	+128 76	+18 39
Green grass + bone meal period.							
June 4-11.....	961 35	16 90	284 29	1,262 54	1,504 86	+242 32	+34 62
" 11-18.....	1,193 85	19 90	268 30	1,382 05	1,520 63	+138 58	+19 79
" 18-24 . . . .	1,074 53	28 10	227 40	1,330 03	1,342 95	+12 92	+1 84

through the long continued use of roughages low in calcium and probably low in the vitamin concerned in calcium assimilation. In such a depleted condition response to an ideal ration would be more readily measured through balance experiments.

On May 14 the metabolism records of the three animals were begun with the use of the unsupplemented green plant tissue, corn silage, and grain mixture such as was used in Experiment I. The details of this experiment were like those of Experiment I. The green plant tissue was secured from the same lawn and was

TABLE V.  
*Record of Calcium Balance and Milk Production of Animal 5.*

Period.	CaO in feces	CaO in urine.	CaO in milk.	Total CaO in excreted.	Total CaO intake	Balance per week	Balance per day	Milk per week
Green grass period.								
	gm	gm.	gm	gm	gm	gm.	gm	lbs
May 14-21 .	350 68	5 12	142 34	498 14	495 43	-2 71	-0 38	152 2
" 21-28 .	338 85	4 88	127 15	471 08	506 83	+35 75	+5 11	135 3
" 28-June 4	477 41	12 64	135 57	625 62	536 22	-89 40	-12 77	142 2
Green grass + bone meal period.								
June 4-11	929 39	12 70	140 05	1,082 14	1,151 62	+69 48	+9 92	156 5
" 11-18	965 94	6 86	131 1	1,103 90	1,179 14	+75 24	+10 75	130 1
" 18-24	870 75	7 30	121 2	999 25	1,017 14	+17 89	+2 55	123 2

*Record of Phosphorus Balance of Animal 5*

Period	P <sub>2</sub> O <sub>5</sub> in feces	P <sub>2</sub> O <sub>5</sub> in urine	P <sub>2</sub> O <sub>5</sub> in milk	Total P <sub>2</sub> O <sub>5</sub> excreted	Total P <sub>2</sub> O <sub>5</sub> intake	Balance per week	Balance per day
Green grass period.							
	gm	gm	gm	gm	gm	gm	gm
May 14-21 .	558 76	6 58	192 09	758 43	787 33	+28 90	+4 13
" 21-28 .	593 82	7 61	166 46	767 99	800 83	+32 84	+4 69
" 28-June 4	564 03	13 62	179 47	757 12	783 85	+26 73	+3 82
Green grass + bone meal period.							
June 4-11 . .	903 21	7 78	171 66	1,082 65	1,244 62	+161.97	+23 14
" 11-18 .	1,013 32	14 95	161 20	1,189 47	1,261 05	+71.58	+10 22
" 18-24. . .	930 65	12 94	139 30	1,082 89	1,121 45	+38 56	+5 51

cut and sampled daily. The ration consisted of 40 lbs. of green plant tissue daily, 20 to 25 lbs. of corn silage, and for 3 lbs. of milk produced daily 1 lb. of a grain mixture composed of 60 parts of yellow corn, 25 parts of wheat bran, and 15 parts of oil meal. On this ration the animals were continued for 3 weeks with quanti-



tative collection of excreta and its sampling for analysis. At the end of 3 weeks 200 gm. of steamed bone meal (same as used in Experiment I) were added daily to the ration of each cow with continued collection and analysis of the excreta and the milk for a period of 3 weeks. The records of these data are presented in Tables IV, V, and VI.

TABLE VI  
*Record of Calcium Balance and Milk Production of Animal 6.*

Period	CaO in feces	CaO in urine	CaO in milk	Total CaO excreted	Total CaO intake.	Balance per week	Balance per day	Milk per week.
Green grass period.								
	gm	gm	gm	gm	gm	gm	gm	lbs
May 14-21	289 11	4 30	156 66	450 07	434 25	-15 82	-2 26	164 5
" 21-28	309 02	6 27	183 13	498 42	493 10	-5 32	-0 76	227 3
" 28-June 4	388 54	3 17	185 02	576 73	536 22	-40 51	-5 79	219 1
Green grass + bone meal period								
June 4-11	895 23	11 83	182 50	1,089 56	1,151 62	+62 06	+8 87	205 0
" 11-18	987 55	7 78	179 40	1,174 73	1,179 14	+4 41	+0 63	207 9
" 18-24	750 08	8 24	134 00	892 32	1,017 13	+124 81	+17 83	149 8

*Record of Phosphorus Balance of Animal 6*

Period	P <sub>2</sub> O <sub>5</sub> in feces	P <sub>2</sub> O <sub>5</sub> in urine	P <sub>2</sub> O <sub>5</sub> in milk	Total P <sub>2</sub> O <sub>5</sub> excreted	Total P <sub>2</sub> O <sub>5</sub> intake	Balance per week	Balance per day
Green grass period							
	gm	gm	gm	gm	gm	gm	gm
May 14-21 . . .	510 19	6 74	193 08	710 01	689 43	-20 58	-2 94
" 21-28	558 54	6 46	220 54	785 54	800 83	+15 29	+2 18
" 28-June 4	522 06	9 50	212 87	744 43	783 85	+39 42	+5 63
Green grass + bone meal period							
June 4-11	845 64	11 42	199 20	1,056 26	1,244 62	+188 36	+26 91
" 11-18	1,013 47	21 95	202 00	1,237 42	1,261 05	+23 63	+3 37
" 18-24	939 44	16 89	139 40	1,095 73	1,121 45	+25 72	+3 67

It is apparent from all the data that green pasturage in the amount and character we allowed and unsupplemented with calcium salts will not maintain calcium equilibrium in cows producing 30 or more pounds of milk daily. It is also probable that the kind of green forage supplied in these experiments was equal

to if not superior to most of the pasturage furnished dairy cattle. It must be remembered that these experiments did not include the possible influence of another potent factor<sup>2</sup> now known to operate in maintaining calcium and phosphorus equilibrium in growing animals; namely, sunlight. All our experiments, including Experiment I, were conducted in the basement of a barn lighted only by diffused light, entering through partly opened windows with a north exposure. What the additional influence of direct sunlight, such as obtains in the summer pasturage of cattle, would be remains to be determined by future experiments. It seems inconceivable that cows of average milk production are in constant negative calcium balance under both summer and winter feeding conditions, but possibly such a condition does generally prevail and accounts for the gradual lowering of milk flow as the period of lactation advances and certain other increasing troubles of dairymen, such as slowness to breed, weakness of offspring, etc. The longer periods of rest between succeeding lactation periods now recognized as essential for high producing dairy cows with their tendency to sterility, may have a direct relation to the need of mineral reserve building.

Phosphorus equilibrium and even storage of this element took place in the unsupplemented period. The plane of phosphorus intake in this period was high—practically double the calcium intake—due to the generous provision of wheat bran in the ration fed. The fact that the phosphorus demands of dairy cows can be met by the use of grains, especially where wheat bran constitutes a liberal portion of the ration, is of considerable practicable moment. It means that the calcium supplement on green pasturage need not necessarily be a phosphate, but can be the cheaper carbonate provided that no evidence accumulates showing that the continual ingestion of calcium carbonate is in any way deleterious. Up to the present time there is no indication of a harmful action through the use of the carbonate for dairy cattle. Whether the remarkable increases in milk production observed by Meigs and Woodward (4) through the feeding of extra amounts of sodium phosphate are to be explained on the assumption that the phosphate served in the maintenance of phosphorus equilibrium

<sup>2</sup> For review of literature on the influence of radiant energy on rickets see Park (3).

of their animals or in some such capacity as Embden and Laquer's (5) lactacidogen remains unexplained.

In the periods in which calcium phosphate was used to supplement the green grasses calcium equilibrium was maintained by a cow producing over 40 lbs. of milk per day (Animal 4), and storage occurred on a lesser daily milk production as in the case of Animals 5 and 6. There was also a considerable apparent storage in the 1st week of the period following the change to the bone meal period, but this was due to the lag in the excretion of calcium following the increased ingestion of this element which is immediately calculated in the balance computations. This same fact is to be seen in Experiment I. The fact that the same animal in negative calcium balance could be changed to a positive balance when one of the factors influencing calcium assimilation was made to vary and the others were held constant—namely, through a constant but liberal supply of the vitamin assisting calcium assimilation but with increased calcium intake—makes it appear certain that at least in these cases negative calcium balances are not to be attributed to a nervous condition of the animal incident to the collection of excreta. This latter theory has been suggested by Meigs, Blatherwick, and Cary (6) as an explanation of negative calcium balances observed by them and by Forbes and his associates (7) in experiments with dairy cows.

In fact the whole phenomenon of the maintenance of calcium and phosphorus equilibrium in milking cows harmonizes with the recent observations on experimental rickets in small animals. These observations postulate the necessary presence in the ration of an ample supply of an organic factor (antirachitic vitamin) assisting calcium and phosphorus assimilation—or its equivalent or supplement in sunlight—and an ample supply in the ration of the elements calcium and phosphorus. Whether the two elements calcium and phosphorus must be in a fairly definite and narrow ratio for optimum assimilation is not finally settled by our data. There were periods of positive balance when the ratio of calcium to phosphorus was approximately 1:2 and other periods of positive balance when the ratio of calcium to phosphorus was approximately 1:1, but our data in this respect are too limited for final conclusions. However, it seems more than probable that a definite ratio of calcium to phosphorus is not so important as the pro-

vision of a liberal plane of each of these elements in the presence of an ample supply of the antirachitic vitamin. We do not anticipate physiological disturbances by the allowance of limited increments of either elements above the minimum requirements.

It is apparent that with cows producing 40 to 60 lbs. of milk per day supplementing the green plant tissue ration with 200 gm. of steamed bone meal per day was not liberal enough to establish definite calcium storage in all cases. It maintained equilibrium in three such cases of high production—Animals 1, 3 (Experiment I), and 4 (Experiment II), but in the case of Animal 2 (Experiment I) negative calcium balance prevailed even in the supplemented period. Had the plane of calcium intake been raised possibly calcium equilibrium could have been obtained in all cases where the green grasses formed a considerable part of the ration.

Without stressing too far a quantitative expression of the amount of calcium oxide required by dairy cattle for maintenance and milk production it would appear from our data that under the conditions of these experiments at least 4 gm. of  $\text{CaO}$  per pound of milk would be required and perhaps a safer figure would be 6 to 8 gm. Approximately the same figure for  $\text{P}_2\text{O}_5$  can be applied. We hope no one will conclude that we are attempting to erect a calcium and phosphorus standard for dairy cattle. This is impossible until all the environmental factors operative in this problem have been studied, and even then only approximate figures should be expected. With some rations deficient in the vitamin concerned in this problem any quantity of calcium supplied would be insufficient.

Phosphorus equilibrium and even storage of this element took place in the periods where bone meal supplemented the ration. This was true with all animals.

#### SUMMARY.

A survey of all our data on the use of green plant tissue as a part of the ration of liberally milking cows supports the idea that the maintenance of calcium and phosphorus equilibrium or the storage of these two elements can be accomplished only when such green plant material as we used is further supplemented with a calcium salt and where the presence of phosphorus is amply pro-

vided, as through the use of wheat bran. The fact that in the case of the cow a generous supply of the antirachitic vitamin can be provided through her liberal capacity for the digestion of bulky materials such as green plant tissue, makes it questionable whether we can expect a more efficient use of calcium and phosphorus through the additional provision of direct sunlight, maintaining at the same time lower planes of intake of the two elements calcium and phosphorus such as exist under practical conditions during summer feeding on unsupplemented pasturage. In fact nothing is known of the relation of light to the maintenance of calcium and phosphorus equilibrium in mature lactating animals. This phase of the subject will be further investigated.

Calcium and phosphorus equilibrium can be maintained in lactating cows when part of the ration is a green roughage, such as alfalfa with its liberal supply of calcium, and when phosphorus is supplied either in organic form such as exists in wheat bran or as a phosphate.

Calcium and phosphorus equilibrium or storage in lactating cows can also be accomplished where the legume has been so cured as to preserve a liberal proportion of its antirachitic vitamin and with provision for plenty of phosphorus in the ration; but with the common grasses of ordinary pasture the calcium content will be too low even when fed in their green state (but out of contact with direct sunlight) to maintain calcium equilibrium in liberal milking cows or even in cows of average capacity. As in the case of nitrogen so in the case of calcium and phosphorus, the lactating animal is extremely wasteful.

As a tentative figure we suggest the provision of 6 to 8 gm. of CaO per pound of milk in the *ideal* ration of a dairy cow. An equal provision of  $P_2O_5$  should be made. An ideal ration must provide an ample supply of the vitamin assisting calcium and phosphorus assimilation.

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## FAT-SOLUBLE VITAMINS.

### XIV. THE INORGANIC PHOSPHORUS AND CALCIUM OF THE BLOOD USED AS CRITERIA IN THE DEMONSTRATION OF THE EXISTENCE OF A SPECIFIC ANTIRACHITIC VITAMIN.\*

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Howland and Kramer (1) in 1920 showed that during active rickets in children the inorganic phosphorus of the blood serum was materially reduced. The calcium content was not necessarily affected; sometimes it also was slightly reduced. Hess and Unger (2) confirmed these observations. Hess and Lundagen (3) furthermore demonstrated a seasonal tide in the inorganic phosphates of the blood parallel to the severity of the rickets. Von Meysenbug (4) and Gyorgy (5) also reported a decrease in inorganic phosphate. In rickety rats Kramer and Howland (6) and Gutman and Franz (7) have reported marked changes in the inorganic phosphates of the blood. Park (8) in his recent review of the etiology of rickets goes as far as to say that "The first detectable signs of rickets are probably a diminution of the inorganic phosphorus or calcium of the blood." Hess and Lundagen (3), however, report that in some cases of rickets no blood changes are demonstrable, and Hess and Unger (2) state definitely that while lowering of inorganic phosphorus generally results, it is not a specific for the disease. This fact we have had abundant opportunity to confirm in our experiments with dogs. From results published in the literature and from data of our own we are convinced that while a reduction of inorganic phosphate need not necessarily precede the incidence of severe

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rickets, sooner or later in the course of the disease, such a reduction results.

When this reduction has taken place, with amelioration of the symptoms and cure of the disease, the composition of the blood is restored to normal. Kramer and Howland (6) showed that this was true for cod liver oil and light of short wave-length, less than 3000 Ångstrom units. They worked with rats. Hess and Gutman (9) showed the same to be true for rickety infants exposed to sunlight.

The above facts suggested to us the possibility of using the restoration of the normal composition of blood in rickety animals as a criterion for the demonstration of the existence of an antirachitic vitamin as distinguished from vitamin A. Funk (10) as early as 1914 suggested the existence of a specific vitamin concerned with the prevention of rickets. Mellanby (11) from numerous experiments with dogs came to the conclusion that this antirachitic vitamin was vitamin A although from the general impression left with the reader he recognized the fact that his experimental data were suggestive rather than conclusive. Hess and Unger (12) presented facts which suggested strongly that the antirachitic substance was not identical with vitamin A, but they were not convinced as to whether this was a vitamin or some other factor. McCollum, Simmonds, Becker, and Shipley (13) made use of the fact that in cod liver oil the antiophthalmic property can be completely destroyed by oxidation. The feeding of such cod liver oil to rats was, however, still able to cure rickets as demonstrated by the deposition of calcium at the hypophyseal junctions. Steenbock and Nelson (14) have shown that cod liver oil in which all vitamin A was destroyed was still able to restore growth in young rats when put upon a ration free from fat-soluble vitamins. They furthermore showed that this restoration of growth was of the same magnitude as that induced by light, a well known antirachitic agent. The experiments reported in the present paper give further support to the thesis that the antirachitic vitamin is distinct from the antiophthalmic substance because such cod liver oil was also found effective in restoring the inorganic phosphate of the sera of rachitic dogs and chickens to the normal.

## EXPERIMENTAL.

The first experiments were carried out with dogs. Five pups with the parent bitch were taken to the laboratory shortly after birth and raised in a dimly lighted room, the bitch being fed a cooked grain mash consisting of equal parts ground white corn and rolled oats and whole milk *ad libitum*. This same ration was also accessible to the pups as soon as their desire for additional food developed.

When 4 weeks old and weighing from 1,170 to 1,340 gm. the pups were weaned and put upon our standard rickets-producing diet. This consists of the aforementioned grain mash autoclaved for 1 hour at 15 lbs. pressure; 200 cc. of centrifuged milk, also autoclaved for 1 hour; 5 gm. of precipitated calcium phosphate; 2 gm. of sodium chloride; and 5 gm. of casein. The grain mash was given *ad libitum*. Distilled water was also given. On this ration we have never failed to produce rickets in any of our animals provided that they were raised on the aforementioned laboratory diet and provided also that they were taken sufficiently young. The importance of the nature of the diet on which the pups are raised and their age at the time that they are put on the experimental diet cannot be overemphasized. We are convinced that the great irregularity in the incidence of rickets in dogs and rats—like that of ophthalmia in these and other animals (15)—is due to the fact that the practise of picking up stray animals or of raising them on laboratory rations of variable composition is all too common. Hess, Weinstock, and Tolstoi (16) have already published data from preliminary observations on the influence of diet on refractoriness to rickets bearing on this point. Our observations, as yet unpublished, confirm their conclusions absolutely. For over 2 years we have been taking precautions designed to eliminate these influences.

After 39 days on the experimental ration all the pups showed symptoms suggesting rickets. Upon being disturbed they would rise hesitatingly, often staggering and turning in a circle as though uncertain on which leg to rest their weight. Once on their feet, their behavior was fairly normal but from this time on, the behavior varied with the individual as shown in the protocols.

The dogs were weighed weekly and observations on their general conditions taken daily, or as necessary. Blood samples were taken after 56 and 80 days on the experimental ration and analyzed for calcium by de Waard's (17) method and for inorganic phosphate by the Bell-Doisy-Briggs (18) method except for the first analyses when the Marriott and Haessler (19) method was still being used.

After 80 days on the experimental ration one dog, Dog 51, had died. Analyses showed that in those living the blood phos-

TABLE I

*Change in Composition of Blood Serum of Dogs with Change of Ration*

Dog No	Change of ration.	Calcium per 100 cc of serum			Phosphorus per 100 cc of serum		
		Before change		After change	Before change		After change
		56 days on basal ration	80 days on basal ration	24 days of aerated cod liver oil administration	56 days on basal ration	80 days on basal ration	24 days of aerated cod liver oil administration
		mg	mg.	mg	mg.	mg	mg
47	1 cc. aerated cod liver oil.	11 33	9 43	13.48	7 21*	4 66	6 29
48	4 " " " "	10 54	9 81	11 30	5 22*	3.30	5 99
49	12 " " " "	10 24	11 22	11 58	5 23*	3 52	6 90
50	None	11 53	10 00		7 57*	4 24	
51	"	10 73			5 78*		

\*Determined by the Marriott and Haessler method (19).

phorus had been reduced materially. Calcium had also been reduced slightly in three out of four of the animals. The condition of the animals was such that a change was imperative. One was given 1 cc. of aerated cod liver oil daily, another 4 cc., and a third 12 cc. This was given with a pipette. One dog was kept as a control. The aerated cod liver oil was part of the same sample used by Steenbock and Nelson (14). As an antiophthalmic it was entirely impotent as there reported.

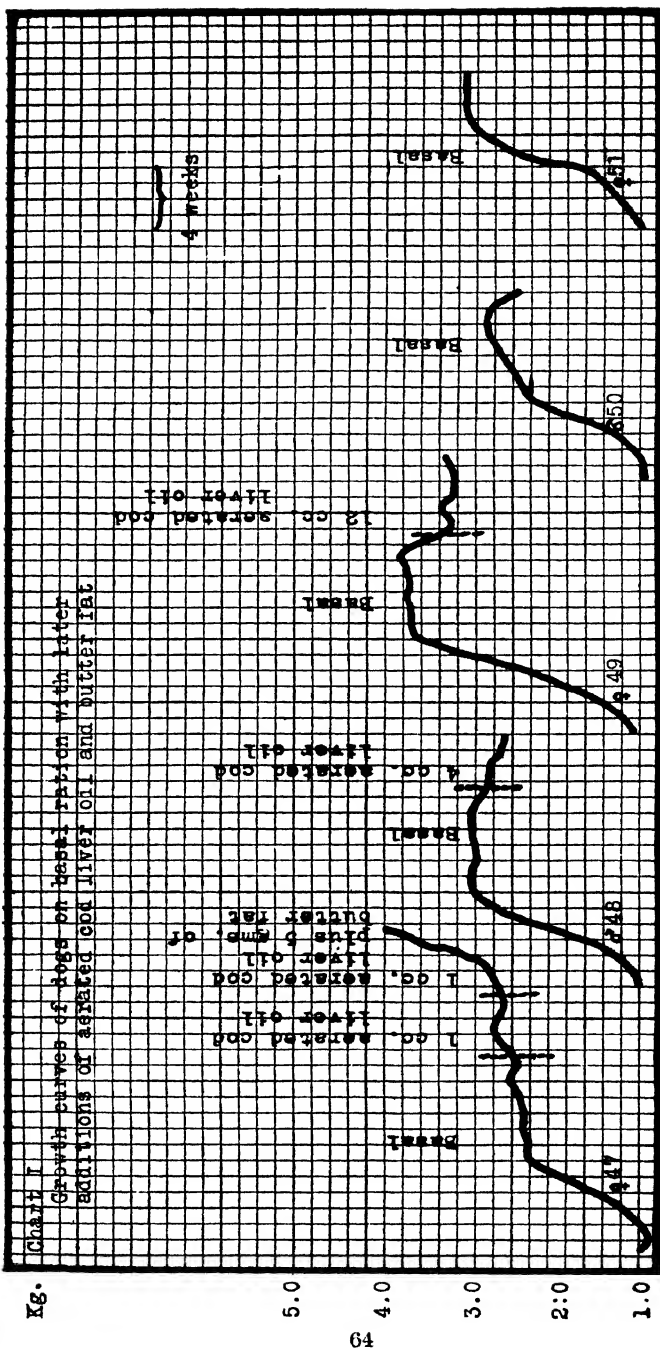
Table I shows the changes that took place in the composition of the blood, first as the animals were continued on the basal ration and second after the addition of aerated cod liver oil to

the diet. The blood was obtained by venepuncture of the external jugular. It was allowed to clot in a refrigerator and the sera were drawn off with a pipette within 24 hours. The sera were centrifuged to remove contaminating corpuscles before pipetting out samples for the analyses.

In all the dogs an increase in calcium occurred with the cod liver oil treatment; but in one instance, that of Dog 49, the increase was not sufficient to be decisive. The inorganic phosphates, however, show conclusively that the aerated cod liver oil, devoid of all antiophthalmic effect, was efficacious even when administered in 1 cc. quantity, as the daily portion, in restoring the inorganic phosphate to approximately normal. Just what the normal is for the dog is difficult to say; apparently, it varies. We have obtained values of 5 to 10 mg. for dogs on what were supposed to be good rations, and Buell (20) has reported values as high as 13.8 mg. (19.2 mg. of  $H_3PO_4$ )—these latter being obtained by the Bloor method (21). It is certain that comparisons can only be made on the same animal and comparing our data in this way, the increase in inorganic phosphate, as determined by the Bell-Doisy-Briggs method, amounted to, respectively, 35, 81, and 96 per cent.

Data were also obtained on the composition of the bones of the animals as represented by the femurs at the time of termination of the experiments. The femurs were dissected out and dried for a week at 96°C. They were then crushed in a screw-press and extracted first with alcohol for 18 hours and then with ether for an equal period of time in a Caldwell extractor. They were then freed from ether, weighed, and ashed in an electric furnace.

Dogs 50 and 51 (both basal animals) gave values of, respectively, 37.81 and 34.38 per cent of ash. Dog 47 first on the basal ration alone, then with this ration plus 1 cc. of aerated cod liver oil, and later with 5 gm. of butter fat gave a value of 41.84 per cent. Similarly, Dogs 48 and 49, receiving first the basal ration and then, respectively, 4 and 12 cc. of aerated cod liver oil, gave values of 43.08 per cent in the case of the former and 40.46 per cent with the latter. All these results point to the ability of the aerated cod liver oil to induce calcium and phosphorus deposition in the bones.



### CHART I.

Chart I shows the weight relations in the animals before and after treatment with the aerated cod liver oil as charted from the weighings, which were made weekly. With the onset of deficiency symptoms, growth was interfered with, which interference the cod liver oil was not able to correct. It is true that a slight response was obtained in the first 2 weeks but this was but a temporary one. These facts strongly suggested the possibility that while these dogs had been getting a sufficiency of the antirachitic vitamin, they were probably not getting sufficient vitamin A. To test out this supposition, Dog 47 was given 5 gm. of butter fat in addition to its cod liver oil. As the curve of growth shows, increase in weight was very pronounced a little over 2 weeks later. The animal began to improve in condition rapidly and was practically normal in behavior and appearance about 4 weeks after the addition of butter fat to its diet was begun.

It is significant that not only was the aerated cod liver oil in excessive dose unable to establish normal conditions completely, but in Dog 48 there developed a purulent rhinitis and in Dog 49, a keratitis, in spite of its use. As both of these conditions are often associated with vitamin A deficiency, they furnish additional proof that the aerated cod liver oil used was free from this dietary essential.

*Protocols on Symptoms and General Behavior of Dogs after Various Intervals on Experimental Ration.*

*Dog 47—39 days* Upon being disturbed in the morning had difficulty in rising. Lay on its side kicking. Upon rising appeared dizzy, then perfectly normal. *46 days* At times dog was normal, then again it staggered when trying to walk. *67 days* Frequent spasms, moves about very little. No marked deformity of legs or thorax. *82 days* No marked change. Dog can still walk and eats fairly well. 1 cc. of aerated cod liver oil given daily. *92 days* Spasms observed daily. *112 days* 5 gm. of butter fat given daily. *114 days* Spasm observed. *132 days* No spasms observed since previous notations. Increase in appetite. *143 days* Dog very active. Acts and appears normal.

*Dog 48—39 days* Rather inactive, staggers when first getting up, then appears normal. *76 days* Condition the same. Quite active. *82 days* Spasms observed frequently. No marked deformities, but appetite is not very good. Given 4 cc. of aerated cod liver oil daily. *94 days* First spasm observed since cod liver oil was given. *106 days* Purulent rhinitis. *114 days* Dead. Lungs practically normal.

*Dog 49*—39 days. Rather inactive, difficulty in rising 61 days. Apparently weak in hind quarters. 67 days. Slight bowing of front legs. 82 days. Can still rise on its front legs, but not on its hind legs. Animal rather thin and thorax somewhat deformed Spasms Given 12 cc. of aerated cod liver oil daily. 90 days Apparent improvement, more lively and spasms only occasionally. 109 days. Still unable to stand on hind legs 112 days. Exophthalmos on right side. Humors released upon application of slight pressure Keratitis as indicated by leucoma. No purulency. Lacrimation of left eye, otherwise normal. 114 days. Leucoma and exophthalmos of left eye 116 days Eyeball not ruptured. Given 10 cc of butter fat daily. 120 days. More active, better appetite 122 days Dead One lung badly infected

*Dog 50*—39 days When first disturbed in the morning, unable to arise, but after staggering and usually turning in a circle its actions became perfectly normal 46 days. Abnormal condition more marked, but no deformity noticeable 67 days Occasional spasms, but more active than Dogs 47, 48, 49, or 51 76 days Deformity of thorax distinctly noticeable Very lethargic, refused to eat 79 days Eyes inflamed 82 days Dead One lung badly infected

*Dog 51*—43 days Walks with difficulty, cries when handled Only animal in series which was continually abnormal 66 days Near death in severe spasm Respiration restored artificially 80 days Found dead though spasms had not been observed for 2 weeks Lungs found badly congested.

The second series of experiments was carried out with twelve White Leghorn chickens weighing from 359 to 655 gm. Grown on the standard ration used for the University flock, they were transferred to our experimental ration of white corn 97, calcium carbonate 2, sodium chloride 1, and skimmed (centrifugated) milk *ad libitum*. This ration has been repeatedly found to lead to a condition of so called leg weakness or rickets in chickens (22) accompanied by a decrease in inorganic phosphorus of the blood.

At the time of transference three chickens, weighing, respectively, 422, 455, and 547 gm., were killed for analysis of blood and bone for control purposes. 4 weeks later three more were killed as additional controls. They weighed 820, 457, and 648 gm. and like the others were gradually losing in weight with the characteristic symptoms of leg weakness. Shortly before, they had weighed, respectively, 880, 542, and 662 gm.

Six of the birds were given cod liver oil with a pipette. This was given daily. One received, 1 cc. of the untreated oil; two, 1 cc. of the aerated oil; two, 3 cc. of the aerated oil; and one, 5 cc.

of the same. Except for these additions the diets were left unchanged.

The aerated cod liver oil used had been prepared by the same method of aeration as that used for the dog experiments. A control sample was tested for vitamin A on twenty-two rats. These had been brought down with ophthalmia in 6 to 7 weeks by maintenance on a purified synthetic ration of alcohol-extracted casein 18, salts 40 (14), 4, agar 2, yeast 6, and dextrin 70. Seven rats had incorporated in their diet 2 per cent of the aerated cod

TABLE II

*Change in Composition of Blood of Chickens with Addition of Aerated and Untreated Cod Liver Oil to Their Basal Ration.*

Chicken No	Dietary addition	Per 100 cc serum.	
		Inorganic phosphorus	Calcium
		mg	mg.
1163	3 cc aerated oil.	6 90	18 06
1190	1 " " "	7 62	16 93
5810	5 " " "	9 20	22 57
1189	1 " untreated oil.	7 02	20 03
5810	1 " aerated oil.	8 80	18 06
1186	3 " " "	7 02	18 06
5801*	None.	4 42	12 50
5806*	"	4 28	9 70
1187*	"	3 58	10 90
1167†	"	4 87	16 0
5825†	"	4 31	13 0
5814†	"	4 63	13 6

\*Controls killed when cod liver oil additions were made.

†Controls killed when the experiment was begun.

liver oil; ten, 4 per cent; and five, 6 per cent. Close observation of the progress of the ophthalmias for 3 weeks showed progressive increase in the severity of the symptoms with death as a resultant in many cases. We believe that we are justified in assuming that all vitamin A had been destroyed in this oil.

Table II shows that the aerated cod liver oil was efficacious in increasing both the inorganic phosphates and calcium of the blood. In the comparison of the birds which received the cod



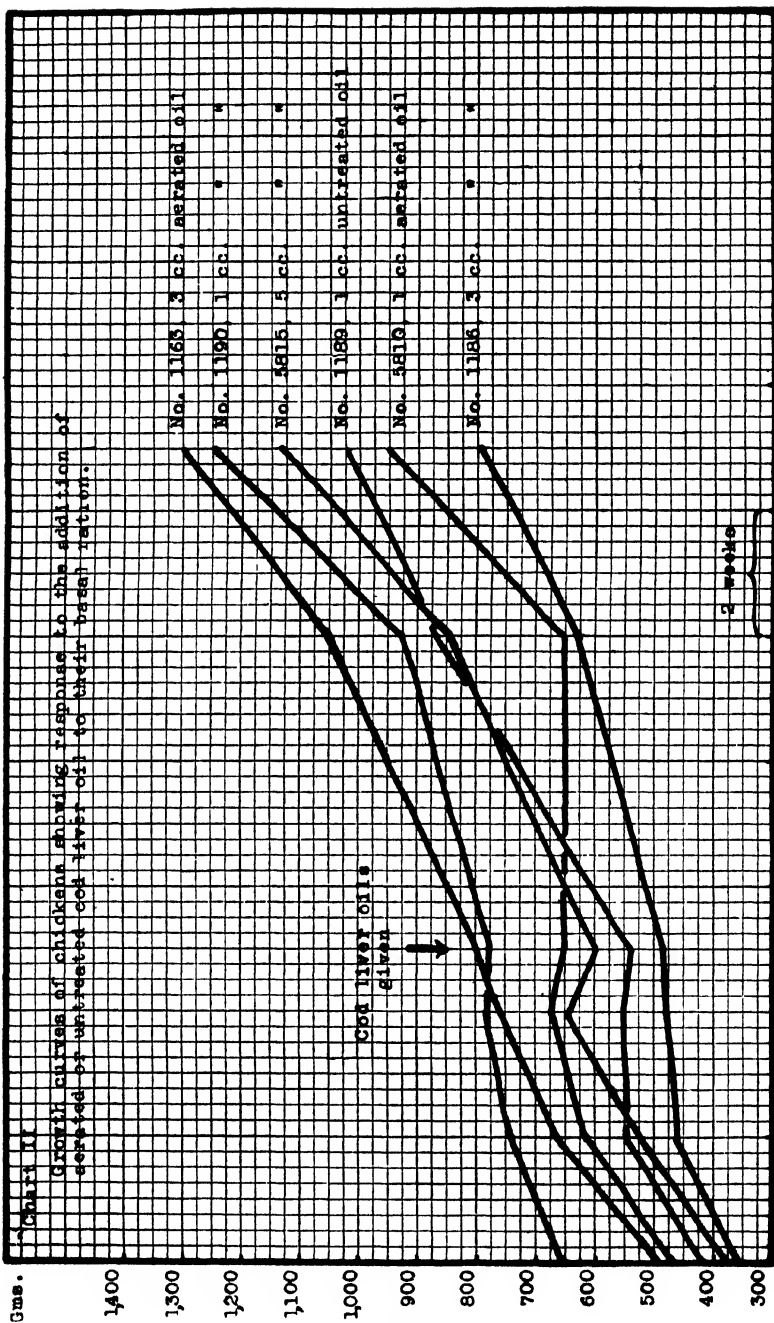


CHART II.

liver oil treatment with the controls, Nos. 5801, 5806, and 1187 carry especial weight because their bloods were analyzed immediately before the cod liver oil administration was begun—the blood of the others had been taken previously. The data show that in the case of both the phosphorus and the calcium the amounts in the sera were practically doubled. This leaves open no question as to the effectiveness of the aerated oil.

With the restoration of the blood of the birds to normal there occurred concomitantly a restoration of growth and amelioration of the symptoms of leg weakness in such of the birds where these prevailed. The growth curves are plotted in Chart II. In spite of the fact that growth was reinitiated by treatment of a cod liver oil free from vitamin A, it is not to be inferred that the bird does not need vitamin A, as had been stated by Sugiura and Benedict (23). In fact, we have unpublished data pointing to the exact opposite which confirms the recent observations of Emmett and Peacock (24). In the first place the white corn-skimmed milk ration contained some vitamin A and in the second place it is probable that the chickens had considerable amounts of this vitamin stored away in reserve. This is probable because the birds were put upon the experimental ration at an advanced age and weight and had abundant opportunity to store this vitamin while still feeding upon the University stock ration.

We believe that this furnishes additional evidence—in contradistinction to the view generally held—that growth is not necessary to produce some, if not all, of the symptoms associated with rickets. For growth to be possible, it appears, as already shown by Steenbock and Nelson (14) for the rat, that the antirachitic factor must be provided in some form or other.

What was shown for the bloods with respect to increase of phosphorus and calcium upon the administration of the aerated cod liver oil was also found true for the bones. At the time that blood samples were taken, the right tibias were also removed and dried at 96°C. They were thoroughly extracted with ether and alcohol, again dried, and then ashed in an electric muffle. The first controls showed an ash content of 44.7 to 47.7 per cent, the second 48.9 to 52.2 per cent. By way of contrast those getting the cod liver oils, taken 13 weeks later, ranged in ash content from 54.6 to 60.2 per cent with the one receiving the untreated oil, standing intermediate at 56.1 per cent.

## SUMMARY.

By the administration of cod liver oil freed from vitamin A by aeration, the inorganic phosphate and calcium of the blood were restored to normal, and the ash content of the bones was increased. These facts are taken as additional justification for the propriety of speaking of the fat-soluble vitamins rather than of a fat-soluble vitamin as it lends support to the idea that the antirachitic vitamin is an entity distinct from vitamin A.

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## FAT-SOLUBLE VITAMINS.

### XV. CALCIUM AND PHOSPHORUS RELATIONS TO GROWTH AND COMPOSITION OF BLOOD AND BONE WITH VARYING VITAMIN INTAKE.\*

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The data presented in this paper had their origin in the desire to determine if the rat is a suitable experimental animal for the study of the factors underlying the deposition of Ca and P in bone. Sherman and Pappenheimer (1) and McCollum, Simmonds, Parsons, Shipley, and Park (2) have shown independently that abnormal bone formation does occur in this species when fed various faulty diets, especially diets low in antirachitic vitamin and either Ca or P. They used as their criteria the occurrence of deformation of the skeleton, especially the vertebræ and ribs, and the presence of abnormal histological pictures as presented by bone sections. Particular emphasis was always placed upon the nature of the observed abnormalities, especially whether or not they represented a duplication of the changes recognized in the infant as rickets. Much valuable information has been accumulated by the use of these methods, yet in themselves they have revealed an imperative need for technique which is more decidedly quantitative in nature. It accordingly appeared worth while to study, first of all, changes in composition of bone and secondly changes in the blood; the former with respect to total ash and the latter with respect to inorganic P and Ca.

Data on the variations in the ash content of bones of rickets, osteomalacia, and kindred bone diseases where the ash content is abnormal are

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already numerous. McCrudden (3) showed the ash content to be reduced in osteomalacia occurring in the human and the horse; Gassmann (4) and Brubacher (5) found the same true for the bones of rachitic children; Telfer (6) and Mellanby (7), for dogs; and Elliot, Creighton, and Orr (8), for pigs. While data on the inorganic composition of rachitic rats as compared with normal individuals have been published by McClendon (9), Sherman and Pappenheimer (10), and McCann and Barnett (11), curiously enough, data on variations in the bones themselves are very limited. McCollum, Simmonds, Kinney, and Grieves (12) have reported that rachitic bones of rats contain from 5 to 15 per cent less of ash—with the exception of the mandibles, which contained from 6 to 8 per cent more—than the normal.

Data on variations in the ash content of bones as the resultant of feeding low calcium diets are also extensive. In most cases these diets have been carefully controlled as far as amount of calcium ingested was concerned, but in the light of present knowledge of nutrition it is questionable whether the results were not sometimes complicated by a deficiency of the anti-rachitic vitamin as well. Roloff (13) in 1874 observed that feeding a dog a diet poor in calcium caused a decided decrease in the calcium and phosphorus of the bones. He found upon analysis that 100 gm of the fresh shoulder-blade bone contained only 6.3 gm of CaO and 4.9 gm of  $P_2O_5$ , while a dog receiving the same diet with added calcium showed 28.6 gm of CaO and 22.1 gm of  $P_2O_5$  per 100 gm of fresh bone. Voit (14) in 1880, feeding dogs diets consisting of meat and fats with and without calcium additions, found that the dried humerus in a normal dog contained 19.13 gm of CaO and 15.1 gm of  $P_2O_5$ , and the one receiving a calcium-deficient diet, 14.85 gm of CaO and 11.87 gm of  $P_2O_5$ . Dibbelt (15), feeding a dog on horse meat, carbohydrate (rice starch), and sodium chloride, found that the ash content of the humerus of a rachitic dog was decreased by approximately one-third compared to that of a normal. Similar results were obtained by Aron and Sebaue (16) and Miwa and Stoeltzner (17).

So far as is known to the authors there is no information available on changes of bone occurring upon feeding low phosphorus diets although the phosphorus content of the ration is one of the important determinants in normal bone calcification (Sherman and Pappenheimer, 1).

Determinations of calcium and phosphorus on blood have been made for 50 years. Many different methods have been used and the results obtained, even on animals from the same species, have not been uniform. It must be remembered, however, that many of the methods used were crude in themselves and in light of our present knowledge cannot be considered free from objectionable features. Most of the determinations, as those of Abderhalden (18), Bunge (19), and others, required a large sample, which, according to our present day methods of experimentation, with smaller animals, are inapplicable and uneconomical. To meet our present requirements, many methods, requiring but small quantities, have been devised rather recently. The most outstanding are those of Bloor (20), Marriott and Haessler (21), and the Briggs (22) modification of the Bell-

Doisy method for phosphorus; and the Marriott and Howland (23), de Waard (24), Kramer and Howland (25), and Kramer and Tisdall (26) method for calcium.

That calcium and phosphorus are not invariable constituents of the blood has been brought out by numerous attempts in the past few years to increase the amount found therein by the injection or feeding of their salts.

Denis and Minot (27) found upon feeding calcium salts, in the form of calcium lactate, to man (6 gm. per day), cats, and rabbits (as high as 1.0 gm. per day) that in most cases it is impossible to increase the concentration of calcium in the plasma by ingestion of calcium salts, but that in cats and rabbits where the initial concentration was low it was sometimes possible to increase it in the plasma.

Clark (28) also attempted to determine, by various means, whether it was possible to produce a definite increase in the calcium content of the circulating blood and if so whether the increase was permanent or transitory. He confined rabbits in metabolism cages and fed them a low calcium diet (equal parts of rolled barley and fresh carrots) with weekly injections, intravenous and subcutaneous, of calcium salts, in doses varying from 19 to 63 mg of calcium. A second group he fed a high calcium diet of alfalfa, barley, and greens, and injected the animals every 5th day with varying doses (200 to 250 mg) of calcium salts. He observed that although most of the injected calcium, 80 to 90 per cent, was rapidly eliminated it was possible to obtain a definite but transitory increase in the calcium content of the blood. These findings were in agreement with the work of Heubner and Rona (29), who used a large number of cats as experimental animals, and with those of von Fenyvessy and Freund (30) who used rabbits. However, by feeding a high calcium diet consisting of 4 parts of rolled barley, 1 part of alfalfa leaves, plus 2 per cent calcium lactate in the diet and the drinking water, Clark found that it was impossible to increase the calcium concentration of the blood of rabbits even though formerly on a low calcium diet.

Phosphates have been injected by different investigators with varying results due to the fact that they show a toxicity. Greenwald (31) found that he could increase the acid-soluble phosphorus of the serum as much as twentyfold without deleterious effect upon the animal. The extent of the increase, before death set in, depended, however, upon the type of phosphate he injected. He showed that the pyrophosphates were twice as toxic as the meta salts, while the ortho compounds exhibited the least effect. Also that the increase of phosphorus in blood by intravenous injections of salts of this element was only temporary. Iversen (32), working with rabbits and guinea pigs, substantiated the findings of Greenwald (31), namely, that pyrophosphates were much more toxic than the meta salts and that the ortho compounds possessed the least action. This investigator also found upon intravenous injection of 100 cc. of 3.6 per cent  $\text{Na}_2\text{HPO}_4(12 \text{ H}_2\text{O})$  a rise of acid-soluble phosphorus from 2.4 to 24.9 mg. per 100 cc of plasma. The increase declined immediately after cessation

of injection, although the decrease was relatively slow and many hours had to pass before a normal concentration was reached, since 3½ hours after completion of injection the phosphorus content was still over twice as much as normal and not until the 7th hour was the normal approached. Similar attempts have been made by other investigators, but there are no experiments, so far as we are aware, that show any permanent influence upon the inorganic phosphate of the blood or serum resulting from the injection or feeding of phosphorus in any form in excess.

Observations of primary importance made by Iversen and Lenstrup (33) and Howland and Kramer (34) quite independently were to the effect that the phosphate phosphorus in the blood of rachitic children was reduced below the normal. Howland and Kramer found that in non-rachitic infants the concentration of calcium was from 10 to 11 mg per 100 cc of serum, and of inorganic phosphorus, about 5 mg. During the period of active rickets the calcium concentration of the serum may be normal or slightly reduced, a result in agreement with that previously reported by Howland and Marriott (35). The inorganic phosphorus of the serum, however, is regularly reduced, in some cases to an extreme degree, for example to 1.0 mg, an observation further substantiated by the findings of Hess and coworkers (36).

With the publication of these results it appeared to us that possibly the blood of rats might show similar changes and a rather extended series of experiments was initiated varying the amounts of calcium, phosphorus, and antirachitic vitamin as supplied by cod liver oil. Before these experiments were completed two publications appeared dealing with this same matter.

In January, 1922, Gutman and Franz (37) reported the production of experimental rickets in rats fed on diets containing various amounts of calcium and phosphorus. Upon an analysis of the whole blood they found in general a reduction in the inorganic phosphate of the blood parallel to the degree of severity of the rachitic lesions. They reported that rats on diets containing 86 mg per cent phosphorus develop rickets with an average for blood phosphate around 3.2 mg. When as little as 75 mg per cent phosphorus were added to the diet, the rachitic lesions failed to appear and the blood phosphorus averaged between 5.5 and 6.0 mg.

In September, 1922, Kramer and Howland (38) reported the chemical examinations of the blood from rats used in the experiments of McCollum, Simmonds, Parsons, Shipley, and Park. They showed that the concentration of the inorganic phosphorus and calcium of the serum could not be made to exceed the concentrations regarded as normal. Further, that when the concentration of either calcium or inorganic phosphorus in the serum was low, it could be increased by increasing the amount of the respective element in the diet. Also, when the inorganic phosphate of the serum was low it could be increased by fasting, the addition to the diet of phosphorus in organic or inorganic form, or of various fats (the fish oils and butter fat), and by exposure to radiations of the requisite quality. The examinations of the blood also indicated that cod liver oil raised the calcium of the serum in the rat when it was low.

In considering the foregoing investigations, we find that considerable work has been done on the relation of diet to the composition of bone and blood, with some evidence as to the possible factors which are operative in affecting the inorganic relations of the body tissues. However, a great deal of the work presented, especially the earlier researches, is subject to criticism in that the conditions under which the work was done were not well controlled. Also, there are no experiments, in so far as the writers are aware, which attempt to show the inorganic relations that exist within the blood and bone tissues of the same animal. With these observations and findings in mind, the authors concerned themselves chiefly with a study of the inorganic relations in the bloods and bones of rats when fed on various deficient diets.

#### EXPERIMENTAL.

Our first determinations consisted of analyses of the bones of rats fed a synthetic ration of purified food constituents to which had been added various amounts of skimmed or whole milk. The synthetic ration consisted of casein 18, salts 32, 4, yeast 2, agar 2, and dextrin 74. The casein was a commercial casein which had been hot alcohol-extracted for 1 week with daily changes of alcohol and then heated in thin layers at 96° for another week. The preparation of the salts and dextrin (39) has already been described. The yeast was a desiccated product previously tested for vitamin B content.

The rats were individually confined in cages measuring 2 feet by 1 foot by 20 inches (height). Shavings were used as bedding. The milk supplied was pipetted out for each rat into heavy porcelain dishes. The basal ration was fed *ad libitum*. Particular attention was paid to the age of the rats when started on account of the ability of the mammal to store the fat-soluble vitamins (40). They were all taken from our stock colony—having been raised on our regular stock ration—at 21 to 24 days of age, and weighed from 45 to 68 gm. This stock ration had the following composition.



*Stock Ration.*

Yellow corn . . . . .	72
Oil meal . . . . .	15
Crude casein . . . . .	5
Alfalfa . . . . .	2
Sodium chloride . . . . .	0 5
Calcium carbonate . . . . .	0 5
Whole milk . . . . .	<i>ad libitum.</i>

Where less than four rats were finally used for the analysis of bone the missing rats had died from the dietary deficiency—four rats having been started in each experimental group. Bone analyses were considered of value on these animals after having been on the experimental ration because, varying with the amount and kind of milk fed, the rats had shown varying degrees of interference with normal locomotion. This partook of the nature of a peculiar shambling gait with more or less stiffness apparent in the hind quarters and what might be assumed as an attempt on the part of the animal to rest lightly on its feet. Such animals were also often observed to object to being handled and when forcibly lifted by the tail would often squeal with pain. Ophthalmias and respiratory disturbances were also evident in many of the animals, sometimes very early in the experimental period.

The rats were killed by means of ether and the two femurs and humeri dissected out from the fresh tissue. They were dried at 96°C. for 2 days, scraped free from adhering tissues, crushed between the jaws of a large pair of pliers, and quantitatively transferred to an extraction thimble for ether-alcohol extraction. This extraction is absolutely necessary previous to the ashing as the bones of animals vary so much in lipoidal and fat content even when taken from animals on the same ration that the accuracy of percentage values is seriously interfered with if omitted. The extraction was carried out in a Caldwell extractor first with hot 95 per cent alcohol for 18 hours and then with U. S. P. 1910 ether, which contained about 2 per cent alcohol, for another 18 hours. After the extraction the bone residue was dried at 96° for 1 hour, carefully weighed, and then incinerated in an electric muffle furnace for 3 hours. The percentage of ash was calculated on the alcohol-ether-extracted basis.

The results of these analyses are presented in Table I. They show a fairly clean cut correlation between the amount of fat-soluble vitamins or at least of milk and the occurrence of motor disturbances on the one hand and deficient ash content of the bones on the other.

The results were sufficiently encouraging to warrant the laying of a broader foundation for further experimental work. First of all it was evident that data on normal animals had to be obtained especially where the age factor—one of the most important ones in bone calcification—was under control. For this, young healthy stock rats 24 days of age and weighing from 45 to 65 gm. were weaned and used immediately or continued on the stock ration until they reached the desired age and weight. In addition to these, three large rats weighing 375 to 425 gm., fully mature though showing no signs of senility and in excellent condition, were likewise killed. Data on their age were, however, not available.

With the above mentioned animals analyses of the blood as well as of the bones were made. For this purpose it was necessary, especially with the younger animals, to pool the blood samples as otherwise not enough was available for the determinations. This was furthermore advantageous as we were desirous of getting average values such as it was contemplated to obtain later on the experimental rations.

To obtain blood samples the rat was anesthetized in a jar with ether and then fastened to a board by spring clips clamped on its legs. Either carotid was laid bare, doubly ligated near the head, and a small weight attached to the proximal ligature. The carotid was then cut between the ligatures and the operating board with the animal placed in a vertical position. By means of small scissors the carotid was then cut into and the blood collected in a centrifuge tube with gentle pressure on the abdomen. No difficulty was experienced in getting good samples. After bleeding, the bones were obtained as already described. Due to the large numbers of animals involved not all the femurs and humeri were analyzed; only four animals were taken for the first three groups, three for the next, and two for the last. The blood samples were allowed to stand overnight in the refrigerator, the serum was then pipetted off, centrifuged to remove con-

*Effect of a Synthetic Diet Supplemented with Milk Products on the Ash Content of Bone.*

Ration	Days on ration	No of animals	Average weight at end of experiment gm	Addition to ration	Bone		Remarks
					Type	Ash per cent	
1649	134	1	95	2 cc. skimmed milk per rat per day	Femur Humerus	53 58 53 44	Ophthalmia observed at end of 14th week. Difficulty in locomotion observed between 9th and 10th week, which increased markedly with age.
1650	134	3	121	5 cc skimmed milk per rat per day.	Femur Humerus	57 85 57 70	Ophthalmia observed in three animals at end of 8th, 15th, and 18th week. A difficult locomotion became apparent between the 9th and 10th week, increasing in severity with age.
1651	134	4	155	10 cc. skimmed milk per rat per day.	Femur Humerus	62 44 62 23	No ophthalmia observed. All animals exhibited a slight stiffness of extremities between 10th and 15th week, again showing normal locomotion after 15th week.
1652	134	2	190	15 cc. skimmed milk per rat per day.	Femur Humerus	62 07 61 34	No ophthalmia observed. Two animals died at end of 9th and 10th week, respectively, with respiratory trouble. Observed a slight stiffness of extremities between 10th and 13th week, which finally improved.

1653	134	4	240	20 cc. skimmed milk per rat per day.	Femur Humerus	65 42 66 21	No ophthalmia observed. Animals appeared to be in good condition, with no difficulty of locomotion. Upon autopsy three of the animals showed congested lungs.
1654	134	4	134	0.5 cc whole milk per rat per day	Femur Humerus	61 82 61 94	No ophthalmia observed. Animals showed signs of sensitiveness to touch and slight difficulty in locomotion at end of 5th, 6th, and 7th week—gradually improving at end of 15th week.
1655	134	3	167	1.0 cc. whole milk per rat per day.	Femur Humerus	65 44 66 24	No ophthalmia or difficult locomotion observed. One animal died at end of 18th week with respiratory trouble.
1656	134	4	214	2.0 cc. whole milk per rat per day.	Femur Humerus	66 02 66 84	No ophthalmia or difficult locomotion observed. Animals in good condition.
1657	134	4	275	5.0 cc whole milk per rat per day.	Femur Humerus	66 79 67 20	No ophthalmia or difficult locomotion observed. Animals in excellent condition.

taminating corpuscles, and analyzed at once for calcium by de Waard's (24) method, and for inorganic phosphate phosphorus by the Briggs (22) modification of the Bell-Doisy method.

The results are tabulated in Table II. The bones show a progressive increase in percentage of ash which had not reached the maximum after 66 days when it totalled 59 to 60 per cent. The analyses within the groups were markedly uniform. None showed a greater variation from the average, plus or minus, than

TABLE II  
*Effect of Size and Age of Animal on the Composition of Blood and Bone.*

Weight of animals	Age	No of animals	Blood		No of animals	Bone		Remarks
			Calcium per 100 cc serum	Phosphorus per 100 cc serum		Type	Ash	
<i>gm</i>	<i>days</i>		<i>mg</i>	<i>mg</i>			<i>per cent</i>	
45-60	24	9	13 64	10 10	4	Femur Humerus	44 97 45 49	Taken at age ready for experiment.
80-100	35	12	11 70	9 42	4	Femur Humerus	49 44 51 31	On stock ration 11 days.
130-175	52	8	11 47	9 34	4	Femur Humerus	56 50 57 12	On stock ration 28 days
200-275	66	6	11 66	9 08	3	Femur Humerus.	59 54 59 31	On stock ration 42 days.
375-425		3	10 50	8 89	2	Femur .. Humerus .	66 15 66 17	Stock rats (no record of age)

5.2 per cent. In most cases the variation was even less than 2 per cent.

The composition of the blood serum appears to be fairly constant in later life but in the very young, both calcium and phosphorus are high. For calcium it has been reported by Meigs, Blatherwick, and Cary (41) that the percentage in the plasma of calves decreases with age; for inorganic phosphorus on the other hand an increase was recorded at least up to 6 months.

When these analyses were completed there were available for analyses rats which had been on experiments designed to test out the comparative availability of calcium and barium salts. These rats had been fed casein 18, agar 2, cod liver oil 2, yeast 2, salt 39 (42), 2.4, and dextrin to 100 as a basal ration, as already reported (42). One group was fed the basal ration only, another, the same with the addition of 0.4 per cent  $\text{CaSO}_4$ , a third, with 0.3 per cent  $\text{Ca}_3(\text{PO}_4)_2$ , a fourth, with 0.8 per cent Ca lactate, a fifth, with 2.0 per cent  $\text{BaSO}_4$ , and the last, with the basal ration modified to the extent that 4 per cent of salt 32 were substituted for salt 39 and 2 per cent of  $\text{BaSO}_4$  were added in addition. All the enumerated Ca salts were added to supply an equal amount of calcium, approximately 0.1 per cent. The barium sulfate was fed at a 2 per cent level on an entirely arbitrary basis to determine its assimilability.

As far as growth is concerned it will be noted from Table III that the behavior of the animals was fairly uniform where Ca salt additions were made to the basal ration irrespective of the form in which it was added,  $\text{BaSO}_4$  appeared to have no depressant effect except when fed without simultaneous Ca additions. This, however, was apparently not due to the barium but due to the fact that in the latter group the animals did not have a sufficiency of fat-soluble vitamin as evidenced by earlier incidence of ophthalmias. Though the same basal ration was fed in both cases the  $\text{BaSO}_4$  group was fed at a later date and slight variability of vitamin stored by the animal (40) as well as decreased vitamin content of the cod liver oil may have been responsible.

We are particularly interested in the fact that with the uniformity of growth and well-being in the animals receiving Ca salts the bones and bloods are also, respectively, fairly uniform in total ash and Ca and P content. Where no Ca was added the ash content is very materially reduced and the Ca of the blood is slightly reduced. The phosphorus on the other hand showed no variation. These facts all appear in harmony with the composition of the ration as it was deficient in calcium, contained plenty of phosphorus, and not a sufficiently reduced content of antirachitic vitamin to depress the inorganic phosphorus.

From the foregoing experiments it was seen how the composition of the bones can be definitely related to age and calcium content

TABLE III  
*Effect of a Low Calcium Diet Supplemented with Various Calcium Salts on the Composition of Blood and Bone.*

Date	Days on ration	No of animals	Average weight at end of experiment	Salt additions	Blood		No of animals	Bone		Remarks
					Calcium per 100 cc serum	Phosphorus per 100 cc serum		Type	Ash	
			gm		mg.	mg.			per cent	
1777	156	4	183	0	9 06	9 09	3	Femur Humerus	54 06 54 08	Low calcium ration. Animals in a fair condition.
1778	102	4	231	0 4 per cent CaSO <sub>4</sub>	10 09	9 52	4	Femur Humerus	64 10 64 62	Animals in good condition.
1781	102	4	234	0 3 per cent Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	11 20	9 41	4	Femur Humerus	61 61 61 84	" " "
1782	109	4	220	0 8 per cent calcium lactate	10 23	8 78	4	Femur Humerus	63 41 63 54	" " "
1856	125	4	86	2 per cent BaSO <sub>4</sub>	9 12	8 41	3	Femur Humerus	41 48 43 11	No ophthalmia observed. All animals exhibited difficulty in locomotion at beginning of 13th week.
1857	125	4	232	2 per cent BaSO <sub>4</sub> + salt 32.	11 89	8 33	3	Femur Humerus	63 56 63 94	Salt 32 at level of 4 per cent in place of salt 39 at 2.4 per cent. All animals in good condition.

of the diet. The variations seemed to be pronounced. In comparison, the composition of the blood appeared to remain remarkably constant, especially considering the extent of change of diet. To study these relations in greater detail with respect to the minimum requirements of Ca and P with the addition of varying amounts of antirachitic vitamin furnished as cod liver oil, was the object of the next two series of experiments.

In the first series, the calcium series, the cod liver oil and calcium were each fed at different levels with the phosphorus remaining constant. In the second series, the phosphorus series, the calcium was kept constant and the cod liver oil and phosphorus were varied. Calcium was furnished as the carbonate, and phosphorus as a neutral mixture of the mono- and disodium and potassium phosphates.

In both cases, four to six rats were kept in a group confined in our standard laboratory cages provided with false screen bottoms. They were given distilled water *ad libitum* and fed a basal diet consisting of casein 18, agar 2, yeast 4, with varying amounts of salts and cod liver oil, and dextrin to make 100. The casein was a commercial casein which had been extracted repeatedly for a period of 7 days with daily changes of 0.2 per cent HCl to remove excess of calcium and phosphates and then with hot alcohol for a week to remove fat-soluble vitamins. We cannot subscribe to the emphatic statement of McCollum, Simmonds, Shipley, and Park (43) that extraction with 0.2 per cent of acetic acid and then drying at about 75° removes all demonstrable amounts of any vitamins and represents a method far preferable to alcoholic extraction. We have employed hot alcohol extraction with great success and in our opinion this represents a more rational treatment than extraction with 0.2 per cent acetic acid, a solvent in which the fat-soluble vitamins are not soluble. In some experiments we have adopted the additional precaution of heating our casein in thin layers in pans at 96° for 1 week as already stated. The agar was a commercial agar purified by extraction with 0.1 per cent HCl for 1 week and then washing with distilled water till neutral. The yeast and cod liver oil were of known vitamin content. The latter was added weekly to the rest of the ration as needed, in order to keep the amount of antirachitic vitamin approximately constant, as we have shown that on standing in contact with the ration pronounced deterioration occurs.



For the calcium series it was necessary to have a salt mixture free from calcium and yet furnishing enough of all other constituents. Such a salt mixture was constructed on the basis of salt 40 (44), omitting both the calcium phosphate and lactate and increasing the sodium and potassium phosphates in order that the phosphate intake might not be disturbed. In adjusting this, both the sodium and potassium phosphates were increased, keeping the ratio between sodium and potassium the same as before. In addition to this the mixture was made neutral by furnishing part of the required phosphorus as the monobasic sodium and potassium salts. This adjustment was made by actual test on the salts available, using brom-thymol blue as indicator. The resulting salt mixture, known as salt 43 had the following composition.

	<i>mole</i>	<i>gm</i>
NaCl	4	233 6
MgSO <sub>4</sub> 7H <sub>2</sub> O	1	246
Na <sub>2</sub> HPO <sub>4</sub> 12H <sub>2</sub> O	1 2	429 2
NaH <sub>2</sub> PO <sub>4</sub> 4H <sub>2</sub> O	0 6	115 2
K <sub>2</sub> HPO <sub>4</sub>	4 8	835 2
KH <sub>2</sub> PO <sub>4</sub>	2 4	326 4
Fe <sub>2</sub> (C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> )·6H <sub>2</sub> O	0 1	59 8
KI	0 01	7 6

It was dried, ground, and fed usually at a 4 per cent level though the amount was slightly varied with differences in moisture content for each quantity prepared. The actual amount fed was based on the intake of the various constituents, outside of calcium, with 4 per cent of salt 40 which has repeatedly been found to produce good growth when conditions were otherwise favorable. Calcium as the carbonate was added from 0.125 to 2 per cent of the ration.

The groups were allowed to remain on their respective rations until such time when it was thought that maximum changes could be expected before secondary complications and death would set in. The animals were bled in groups, as before, and the blood from each lot was pooled and the serum analyzed, as previously stated. For bone analysis, the two femurs and humeri were dissected out from representative animals of the groups with 0, 1.0, and 2.0 per cent CaCO<sub>3</sub> additions. In case the group con-

tained four animals, two (usually the highest and lowest in weight) were saved; while if six rats appeared in the group, three (lowest, medium, and greatest in weight) were saved and treated as formerly described.

In the phosphorus series the animals received a similar ration except, of course, for the salt ingredients. In order to obtain comparable results the modifications were made on the basis of salt 43, introducing calcium in sufficient amount as the carbonate. As a phosphorus-free mixture was desired for the basal mixture, the sodium and potassium phosphates had to be withdrawn, but as it was not thought desirable to reduce the intake of sodium and potassium or change the proportion in which they occurred in the ration the amounts withdrawn as phosphates were replaced as chlorides. Furthermore, to prevent excessive salt concentration with addition of the phosphates in the series the amount of chlorides added was adjusted to keep the sodium and potassium constant. To meet all these conditions three separate salt mixtures were compounded. Salt 44 contained the basal constituents, salt 44a the sodium and potassium chlorides, and salt 44b the neutral phosphate mixture as used in salt 43. They had the following composition.

*Composition of Salt Mixtures.*

<i>Salt 44.</i>	<i>Salt 44a.</i>	<i>Salt 44b.</i>	
Sodium chloride 23 38	Sodium chloride 11 69	$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 42 92	
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 24 60	Potassium chlo-	$\text{NaH}_2\text{PO}_4 \cdot 4\text{H}_2\text{O}$ 11 52	
$\text{CaCO}_3$ 45 00	ride 59 56	$\text{K}_2\text{HPO}_4$ .... 83.52	
Ferric cit-		$\text{KH}_2\text{PO}_4$ 32 64	
rate $6\text{H}_2\text{O}$ .. 5.98			
Potassium io-			
dide 0 16			

In practise salt 44b had to be dried so it could be ground. The same object was accomplished by drying the disodium phosphate at room temperature because in the oven a melt resulted which made desiccation without excessive heating difficult, if not impossible. The air-dried phosphate was then mixed with the others. Drying reduced the weight of 170.6 gm. of the mixture 22 gm. which was allowed for in the amount of the salt weighed out for 100 parts of the ration.

Six different phosphate additions, viz. 0.53, 1.06, 1.59, 2.12, 2.65, and 3.18 gm. per 100 gm. of ration, were made and fed in addition to the basal ration without phosphate addition. Corresponding to the above groups there were fed 2.28 gm. of salt 44a for the basal lot and 2.28, 1.90, 1.52, 1.14, 0.76, and 0.38 gm., respectively, for the others with the exception of the last where no additions were necessary as the required sodium and potassium were furnished by the phosphate.

In the calcium series, the first observations of interest are the differences in growth resulting on the various rations as the amount of fat-soluble vitamins and Ca is varied. Chart I shows that even with the addition of abundant calcium, growth is impossible and in fact does not vary with the different lots. The Ca content of the basal ration had been reduced by the processes of extraction to 55 mg. for 100 gm. of ration. The growth on this was of the same order of magnitude as the others, but it is not shown on the chart because the animals were killed at the end of 2 weeks for blood and bone analyses. It is noteworthy that while little growth resulted in any case most of the rats maintained themselves from 6 to 8 weeks before signs of vitamin A deficiency appeared; in fact, only ten instances of ophthalmia were observed by the time that the animals were killed. This indicates that vitamin A deficiency was not the dominant deficiency, but that probably lack of the antirachitic vitamin was responsible for the lack of growth because Steenbock and Nelson (44) have shown that illumination with ultra-violet light will restore normal growth in such rats.

Chart II presenting the growth of the rats on the basal ration when supplemented with 0.1 per cent of cod liver oil shows that if sufficient Ca is added normal growth can be obtained for at least 10 weeks. Up to 1.0 per cent of  $\text{CaCO}_3$  addition, giving with the basal ration an intake of 455 mg. of Ca per 100 gm. of ration, the amount of growth increased with calcium additions. Beyond that, if as much as 2 per cent of  $\text{CaCO}_3$  were added, growth was again definitely depressed. This was the experience uniformly found in all the groups. Whether this is dependent on acid-base balance or actual depressant action of excess calcium we are unable to say, but it apparently is a fact without question as we have observed similar occurrences upon feeding such amounts of  $\text{CaCO}_3$  in other series of experiments.

Charts III, IV, and V bring out relations which are apparently significant. They indicate, in comparison with Chart II, that while with the same cod liver oil intake increased growth may

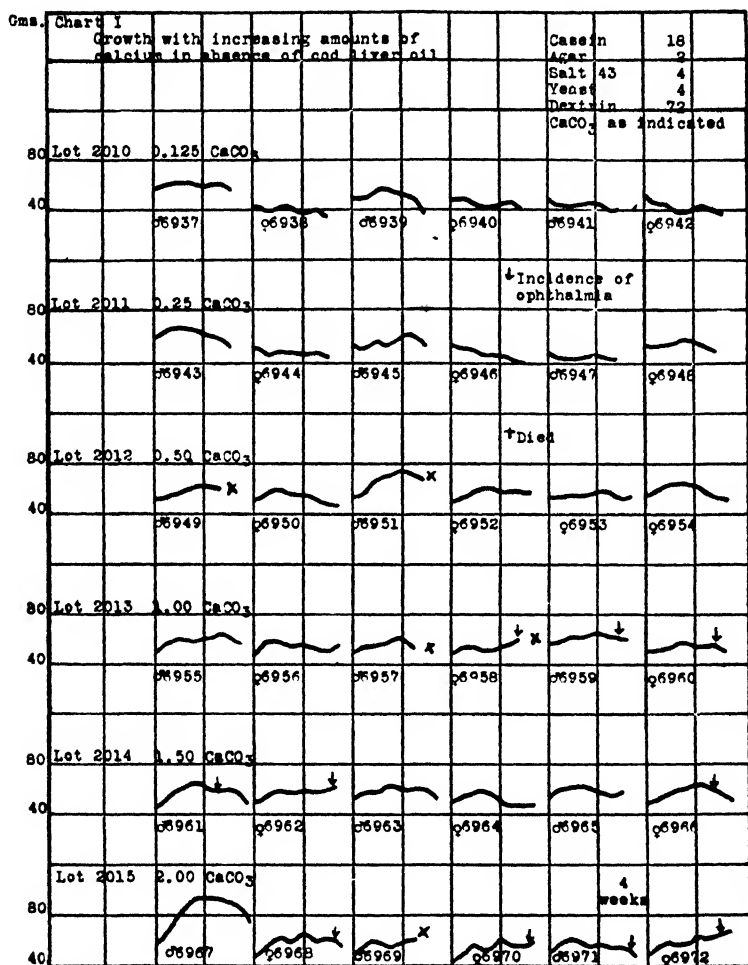


CHART I.

result as the calcium intake is increased, the same result may be obtained by keeping the calcium constant and increasing the cod liver oil. This shows that vitamin requirement and Ca require-

ment are quantitatively interdependent. A suggestion of this fact was obtained in the experiments of Steenbock, Hart, Sell,

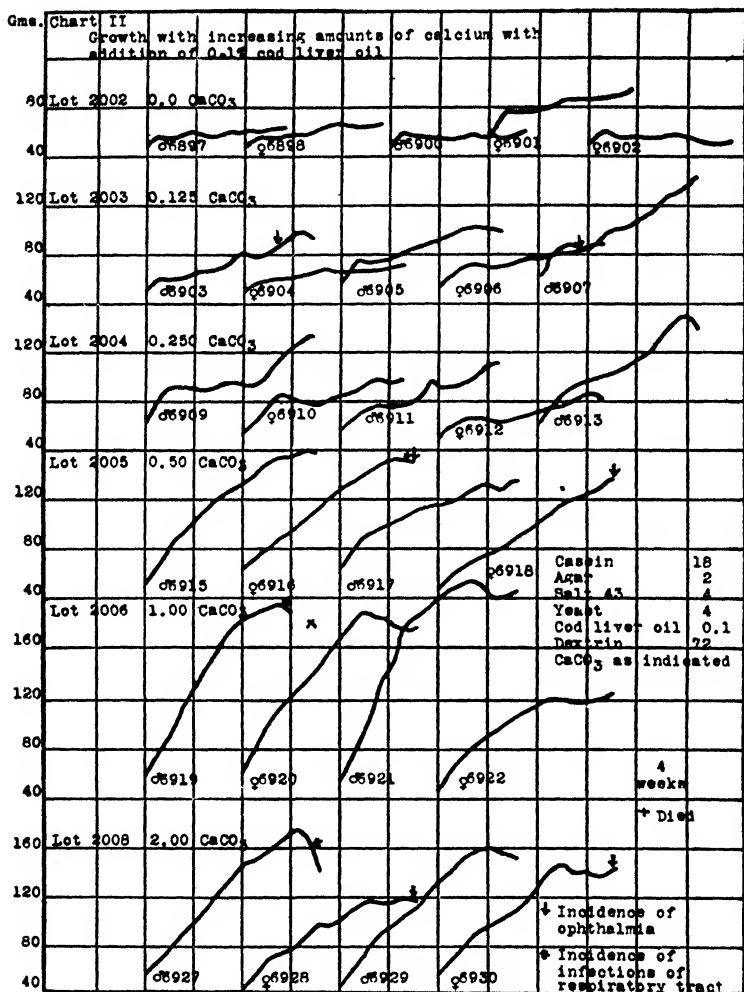


CHART II.

and Jones (42) where it was observed that on a uniform cod liver oil intake, irregularities in growth were observed with low calcium but not with high calcium rations. At that time the fore-

going explanation could only be suggested because the high calcium rations were made up more frequently and therefore the vitamin content undoubtedly suffered less deterioration on standing. This objection does not hold in the present trials because all rations were made up weekly.

In this connection it is interesting to speculate if after all it is not possible that many of the beneficial effects of Ca feeding have

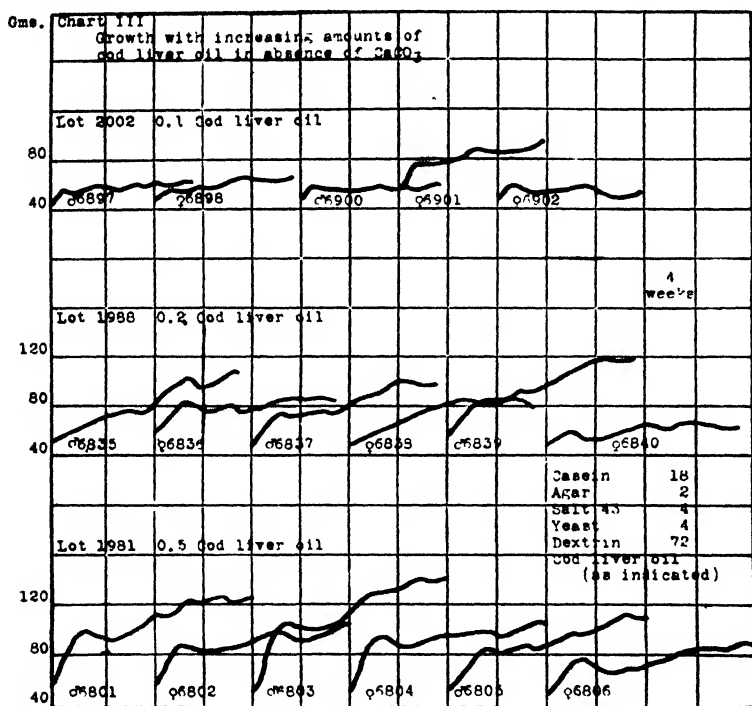


CHART III

had their origin in calcium vitamin relations. With low vitamin content of the ration, increased calcium ingestion may be decidedly beneficial when in reality the deficiency may primarily be that of the antirachitic vitamin. We refer here particularly to the single plant ration experiments of Hart and coworkers (45) where, upon feeding wheat straw and oat straw to cows, disturbances in reproduction were corrected by Ca additions.

In Table IV are shown the variations in composition of the bones. They can be interpreted best by reference to Tables V and VI which give the ages and weights of the animals when the samples were taken. As we have already indicated how the ash content varies with the age of the rat, naturally the ash content of

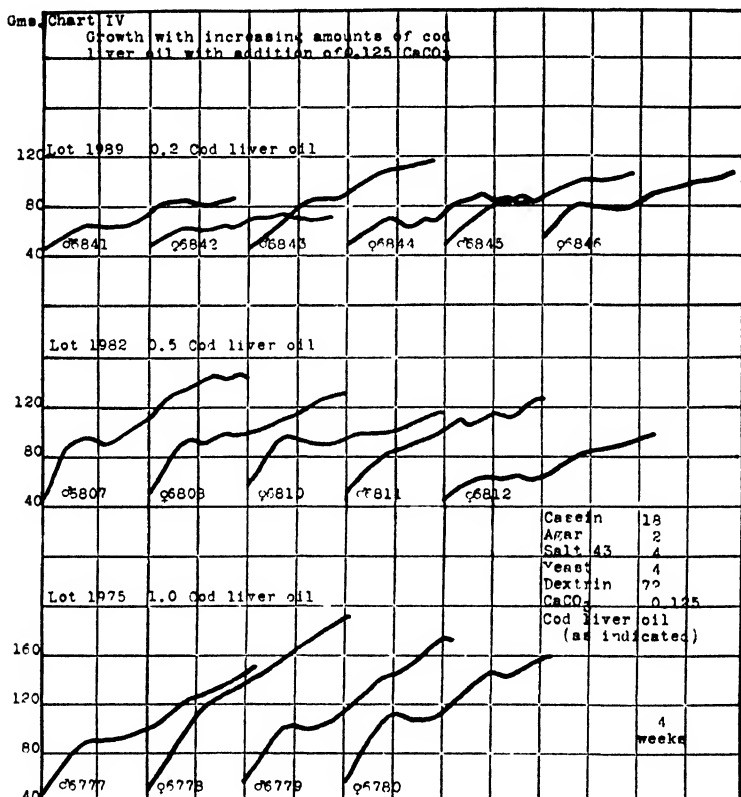


CHART IV.

the series not receiving cod liver oil additions would be low, because the samples were taken after 2 to 8 weeks feeding while the others with two exceptions were taken after 13 to 17 weeks. The data are limited, yet they show that in the absence of additional vitamin, Ca additions had only a minimal effect in increasing mineral deposition in the bones. These rats were kept as long as

possible on the experimental ration, but in the absence of growth, Chart I, the ash content remains practically what it must have been or with a possible slight reduction from what it was when these animals were started on the ration. See Table II.

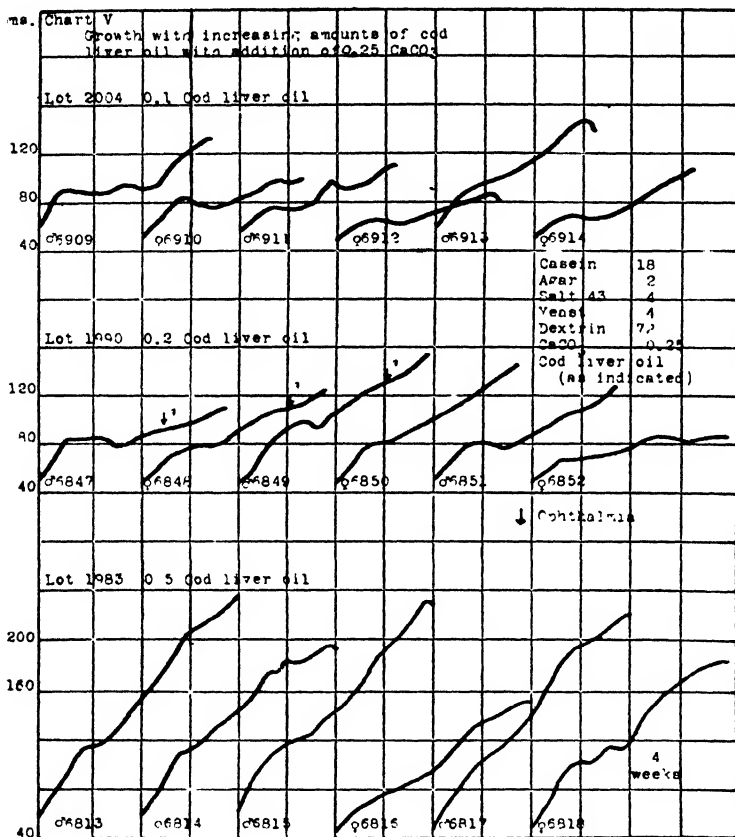


CHART V.

Absence of Ca with excess of cod liver oil has the same effect. Though these animals were taken after 2 to 17 weeks on the ration with a variable cod liver oil intake of 0 to 4 per cent the ash content was remarkably uniform. With 2 or 4 per cent of cod liver oil after 7 weeks the ash content was the same as it was at 2 weeks with no cod liver oil addition or at 17 weeks with 1 per cent of



TABLE IV  
*Percentage of Ash in Bones (Alcohol-Ether-Extracted) from Animals in the Calcium Series Where the Phosphorus Remained Constant with Varying Calcium and Cod Liver Oil.*

Calcium carbonate additions.	Ca and P in ration	Approximate ratio of Ca to P in ration	Type of bone	Percentage of cod liver oil additions							
				0	0.1	0.2	0.5	1.0	2.0	4.0	
0	Ca. . 55	1:12	Femur	39 88*	38 38†	38 55	38 88	41 02	38 83	37 80	
	P. . . 655			42 66	39 92	42 60	41 81	42 26	39 56	42 02	
1 00	Ca. . . 455	1:1.5	Femur . .	43 72	62 79	57 67	63 26	63 42	63 49	62 41	
	P. . . 655			45 39	63 20	58 34	63 23	64 45	63 63	63 19	
2.00	Ca. .... 855	1:0.75	Femur . .	45 02	63 83	59 31	61 14	63 55	63 38	60 01	
	P. .... 655			46 87	64 01	59 30	61 81	63.50	64 90	60 08	

\* Taken at end of 2 weeks.

† Radiated for 10 minutes daily (except Sunday) with ultra-violet light for last 11 days of experiment.

cod liver oil. Yet 1 per cent of cod liver oil allows perfectly normal growth to take place for at least 4 months when 0.25 per cent of  $\text{CaCO}_3$  is added, as shown by the final weight of the animals taken when killed. Table VI.

TABLE V

*Showing the Time (in Weeks) the Various Groups Were Allowed to Remain on Their Respective Rations—Rations in the Calcium Series.*

Calcium carbonate additions per cent	Percentage of cod liver oil additions						
	0	0.1	0.2	0.5	1.0	2.0	4.0
0	2	13	15	16	17	7	7
0.125	6	13	15	16	17	16	15
0.250	6	13	15	16	17	16	16
0.500	7	14	15	16	17	16	16
1.00	7	14	15	16	17	16	16
1.50	7	14	15	16	17	16	16
2.00	8	14	15	16	17	16	15

TABLE VI

*Showing Average Weight of the Rats (in Grams) at the Time of Bleeding in the Calcium Series.*

Calcium carbonate additions per cent	Percentage of cod liver oil additions						
	0	0.1	0.2	0.5	1.0	2.0	4.0
0	45	70*	91	113	108	98	96
0.125	41	94	97	145	165	164	155
0.250	46	102	124	202	194	206	195
0.500	50	145	156	206	210	222	205
1.00	59	167	132	190	249	197	171
1.50	57	170	138	164	198	185	159
2.00	62	135	120	163	198	193	152

\* Radiated.

With both calcium and cod liver oil additions bone calcification was apparently normal when 1 per cent of  $\text{CaCO}_3$  and 0.1 per cent of cod liver oil were added. The ash content was as high as when these constituents were increased, respectively, to 2 and 4 per cent.

In Table VII are shown the data on the composition of the blood with respect to calcium and phosphorus. Here, as in case of the bones, Ca additions without the simultaneous addition of vitamin had no effect. (This also harmonizes well with the observed growth, Chart I, which was remarkably uniform throughout this series.) With vitamin additions, however, the situation changes. With 0.1 per cent cod liver oil the maximum calcium content is reached with 0.5 gm. of  $\text{CaCO}_3$ , and as the amount of cod liver oil increases there is indicated a tendency for the required amount of carbonate addition to be decreased. These observations harmonize with the growth relations already discussed which brought out the fact that no amount of Ca added to the basal ration without vitamin addition was effective in increasing growth, but when cod liver oil was added progressively in increasing amounts, less and less Ca was required for normal growth. To this extent there is established a parallelism between growth and Ca concentration of the blood. Apparently an excessively high Ca concentration cannot be produced by excessive Ca and cod liver oil ingestion.

The inorganic phosphorus of the blood apparently is more stable under the experimental conditions adopted which probably is to be explained by the fact that the phosphorus content of the diet was sufficient even in the first experiments of the series. In general, however, inspection of the table reveals a tendency for the phosphorus to be reduced as the calcium increases. This suggests a depressant effect of the calcium on the solubility of the inorganic phosphates. As already pointed out, bone analyses did not show similar variations so that no matter how suggestive our data may be we have no absolute evidence that variation in ratio of Ca to P is of primary importance in bone formation as maintained by McCollum, Simmonds, Shipley, and Park (43). Indirectly this undoubtedly has some effect, but the relation of mere concentration of elements in the blood stream to Ca and P deposition in bone is far from simple. When first pointed out by Howland and Kramer (34) and Iversen and Lenstrup (33) the reduced P content of blood serum in rickets offered a suggestion as to the possible mechanism of poor bone formation, but skeletal changes are not always simultaneously incident with changes in composition of the blood. In fact, the concentration of P as inorganic phosphates may be normal in very severe rickets.<sup>1</sup>

<sup>1</sup> Unpublished data.

TABLE VII.

*Blood Analysis.**Calcium Series; i.e., Varying Calcium and Cod Liver Oil with Phosphorus Constant.*

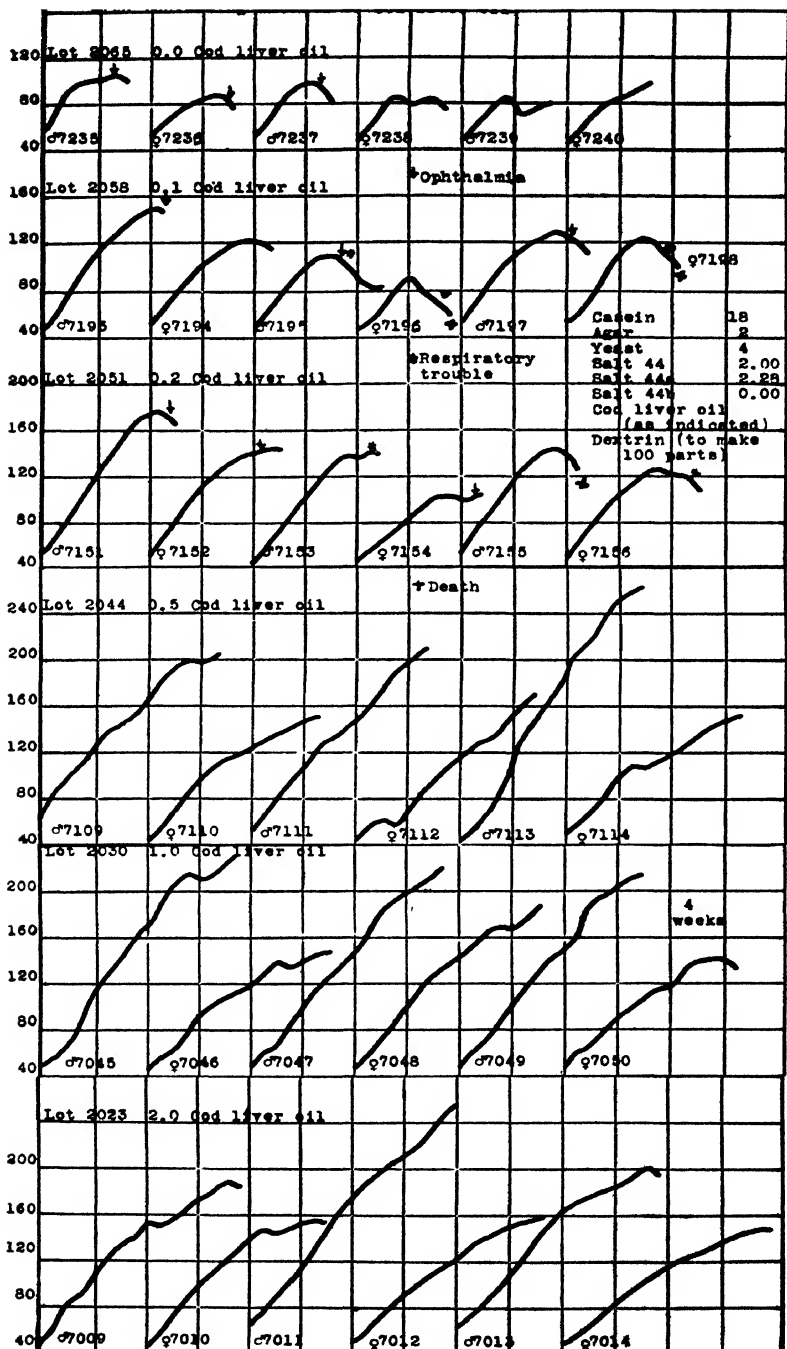
Calcium carbonate additions	Ca and P in 100 gm ration	Approximate ratio of Ca to P in ration	Percentage of cod liver oil additions						
			0	0 1	0 2	0 5	1 0	2 0	4 0
Milligrams of calcium per 100 cc. of serum.									
per cent	mg		mg	mg	mg.	mg.	mg	mg	mg.
0	Ca . . . 55	1:12	10 95*	10 36†	9 30	6 24	8 31	10 09	8 53
	P . . . . 655								
0 125	Ca . . 105	1:6+	8 75	8 26	9 57	8 60	9 69	12 04	11 34
	P . . 655								
0 250	Ca . . 155	1:4+	9 62	10 22	10 26	11 24	12 46	12 60	12 88
	P . 655								
0 500	Ca . . 255	1:2 5+	7 87	11 65	12 48	11 52	12 18	11 76	12 60
	P 655								
1 00	Ca 455	1:1 5+	7 29	11 51	12 48	11 66	11 36	11 48	12 32
	P 655								
1 50	Ca 655	1:1	8 74	11 65	12 20	11 79	12 04	12 04	13 16
	P 655								
2 00	Ca . 855	1:0 75+	Lost.	11 65	12 48	11 79	11 77	12 32	12 74
	P 655								

Milligrams of inorganic phosphorus per 100 cc of serum.

0	Ca . . . 55	1.12	10 63*	9 52†	9 75	10 10	10 20	9 90	9.12
	P . . . . 655								
0 125	Ca . . . 105	1 6+	8 93	9 90	9 61	9 70	9 80	8 47	7 87
	P . . . . 655								
0 250	Ca . . . 155	1 4+	9 09	9 52	9 75	9 00	9 61	8 33	8 06
	P . . . . 655								
0 500	Ca . . . 255	1 2 5+	10 00	8 13	8 26	8 06	9 17	7 87	7 20
	P . . . . 655								
1 00	Ca . . . 455	1 1 5+	8 77	8 47	9 75	7 75	8 33	7 19	7 93
	P . . . . 655								
1 50	Ca . . . 655	1 1	6 33	9 75	8 54	7 40	7 73	8 78	7 66
	P . . . . 655								
2 00	Ca . . . 855	1 0 75+	Lost	8 20	8 93	7 19	7 63	8 09	7 90
	P . . . . 655								

\* Radiated 10 minutes daily with ultra-violet light for the last 11 days of the experiment.

† Taken after being on diet for only 2 weeks.



Growth curves of the animals in the phosphorus series are shown in Charts VI and VII. Chart VI shows that as the amount of cod liver oil is increased up to an intake of 0.5 gm. per 100 gm. of ration, practically normal growth is obtained. This indicates that with casein as the protein, fed at a level of 18 per cent of the ration, the phosphorus cannot be sufficiently reduced to cause

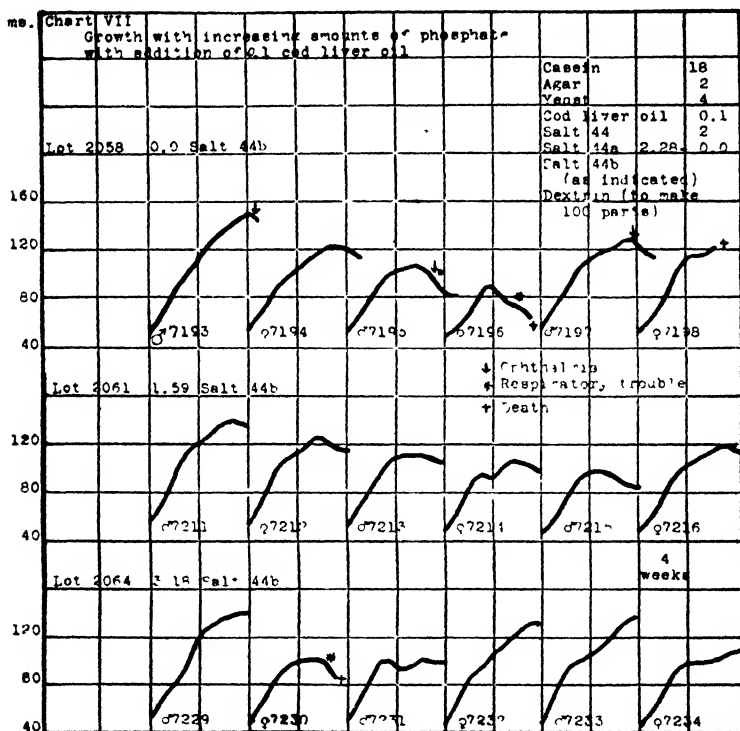


CHART VII.

nutritive failure. Even with lower levels of cod liver oil intake, increase of phosphate does not lead to increased growth. This is shown in Chart VII. In fact, on the basal ration without cod liver oil additions the highest phosphate addition showed depression of growth as compared with no addition. Both these groups were started at the same age and within 4 gm. of the same average

weight, yet the former (for the six animals in the first 4 weeks) showed an average gain of 15 gm. as compared with 33 gm. for the latter.

The bones of the animals in the phosphorus series show nothing of significance with respect to ash content (Table VIII). There is a general tending to an increase in ash content with increase of cod liver oil additions, but this is undoubtedly to be explained by the relative ages of the animals as shown in Table IX. Those with little or no cod liver oil in the rations had to be killed early, due to rapid failure from ophthalmia and respiratory infections.

TABLE VIII

*Percentage of Ash in Bones (Alcohol-Ether-Extracted) from Animals in the Phosphorus Series Where the Calcium Remained Constant with Varying Amounts of Phosphorus and Cod Liver Oil.*

Salt 44b additions	Ca and P in 100 gm ration	Ap- prox- imate ratio of Ca to P in ration	Type of bone	Percentage of cod liver oil additions					
				0	0 1	0 2	0 5	1 0	2 0
<i>per cent</i>	<i>mg</i>								
0	Ca 447	1.0 12	Femur	39 78	57 86	57 32	61 29	60 32	58 94
	P 55		Humerus.	43 36	57 20	57 90	61 54	61 17	59 76
1 59	Ca 447	1:0 8	Femur	50 24*	57 34	57 95	61 52	59 11	62 72
	P 355		Humerus	50 85	58 02	58 51	62 12	61 19	63 23
3 18	Ca 447	1.1 5	Femur	44 33	57 97	58 61	59 15	61.17	63 21
	P 655		Humerus	45 58	57 99	59 63	60 59	61 67	63 37

\* Radiated for 10 minutes daily (except Sunday) with ultra-violet light for the last 21 days of the experiment

Blood analyses also are not especially significant, Table XI. The inorganic phosphorus is practically constant except where no phosphates or cod liver oil were added. Calcium is consistently depressed where no cod liver oil was added, with apparently the greatest depression where the most phosphate was given. These results support the fact that casein carries enough phosphorus for maintenance of normal composition of the blood as well as growth. This is not to be considered the maximum, however, as is indicated by the results obtained by exposing the rats to ultra-violet light. The phosphorus promptly increased 18 per cent over the highest

value where no cod liver oil was added in the course of 3 weeks. With this the calcium also increased and Table VIII shows an increased percentage of ash in the bones. All these changes were accompanied by increased growth of an average of 13 gm. in five

TABLE IX

*Showing the Time (in Weeks) the Various Groups Were Allowed to Remain on Their Respective Rations on the Phosphorus Series.*

Salt 44b additions	Percentage of cod liver oil additions					
	0	0 1	0 2	0 5	1 0	2 0
<i>per cent</i>						
0	6	9	10	13	14	15
0 53	6	8	10	13	14	15
1 06	6	8	9	13	14	15
1 59	6	8	9	13	14	15
2 12	6	8	10	13	14	15
2 65	6	8	9	13	14	15
3 18	6	8	9	13	14	15

TABLE X

*Showing Average Weight of Rats at Time of Bleeding in the Phosphorus Series.*

Salt 44b additions	Percentage of cod liver oil additions					
	0	0 1	0 2	0 5	1 0	2 0
<i>per cent</i>						
0	85	115	126	186	188	191
0 53	71	119	117	204	208	219
1 06	73	121	126	194	185	184
1 59	82*	110	123	181	176	170
2 12	82	126	127	180	183	234
2 65	72	111	141	174	187	191
3 18	68	122	149	160	198	168

\* Radiated.

of the six animals—after growth was stationary—before complete failure due to vitamin A deficiency ensued. The sixth animal was already afflicted with respiratory infections before radiation was started and therefore no response was effected.



TABLE XI.

*Blood Analysis.**Phosphorus Series; i.e., Varying Phosphorus and Cod Liver Oil with Calcium Constant.*

Salt 44b addi- tions	Ca and P in 100 gm. ration	Approx- imate ratio of Ca to P in ration	Percentage of cod liver oil additions					
			0	0 1	0 2	0 5	1 0	2 0
Milligrams of calcium per 100 cc. of serum.								
per cent		mg.		mg	mg	mg	mg	mg
0	Ca	447	1:0 12	8 96	11 06	11 20	12 18	12 32
	P .	55						
0 53	Ca.	447	1:0 3	9 39	11 76	12 32	11 91	12 05
	P . .	155						
1 06	Ca.	447	1:0 5	7 28	Lost.	11 57	12 05	11 91
	P .	255						
1 59	Ca.	447	1:0 8	10 64*	11 90	12 32	11 91	12 18
	P	355						
2 12	Ca	447	1:1 0	7 14	12 04	12 60	11 63	11 91
	P	455						
2 65	Ca	447	1:1 2	7 28	12 04	12 74	11 91	12 05
	P	555						
3 18	Ca	447	1:1 5	6 86	11 76	12 32	11 36	11 91
	P	655						
Milligrams of inorganic phosphorus per 100 cc. of serum.								
0	Ca	447	1:0 12	6 80	8 19	8 54	7 93	9 00
	P	55						
0 53	Ca.	447	1:0 3	8 65	8 29	9 09	8 47	8 54
	P . .	155						
1 06	Ca.	447	1:0 5	8 85	Lost	7 75	8 85	8 47
	P.. .	255						
1 59	Ca.	447	1:0 8	10 41*	8 26	9 43	8 47	9 53
	P	355						
2.12	Ca	447	1:1 0	8 17	9 90	9 09	7 35	8 19
	P .	455						
2 65	Ca.	447	1:1 2	8 80	10 00	9 17	8.33	7.14
	P .	555						
3 18	Ca....	447	1:1 5	8 54	9 65	9 17	9 26	7.46
	P . .	655						

\* Radiated 10 minutes daily (except Sunday) with ultra-violet light for the last 21 days of the experiment.

## SUMMARY.

On a synthetic ration of purified food constituents deficient in fat-soluble vitamins poor calcification of bone occurred in rats even with the addition of 15 cc. of skimmed milk or 0.5 cc. of whole milk per rat per day. 20 cc. of skimmed milk or 1 cc. of whole milk on the other hand allowed normal calcification.

Data were accumulated on the variation in composition of blood and bone with age in the rat preliminary to further studies. It was found that at weaning time at an age of 24 days and 45 to 60 gm. in weight the femurs and humeri contained approximately 45 per cent ash. At 66 days of age when the weight had increased to 200 to 275 gm. the percentage of ash had increased to 59. Maximum values were not attained until much later when the animals weighed 375 to 425 gm.; the ash content then totalled 66 per cent. The blood phosphates and calcium were found slightly higher in the very young than in the older animals.

On a basal ration containing 105 mg. of calcium and 655 mg. of phosphorus per 100 gm. of ration marked increase of growth was obtained on the one hand with calcium additions and on the other hand also with the addition of fat-soluble vitamins as found in cod liver oil. This indicates the existence of a quantitative relation between vitamin and calcium in effecting the assimilation of the latter and raises the question if some of the beneficial results recorded in the literature on the feeding of calcium salts may not have been due to mass action of the calcium counteracting the effects of a vitamin deficiency.

In the absence of normal growth there is shown a general tendency for reduced calcium content of the blood and ash content of the bones. The phosphorus of the blood was found more constant, but apparently as the calcium increases there is a tendency for the phosphorus to be depressed.

With casein as the protein fed at a level of 18 per cent of the ration the phosphorus requirements of the rat appear to be complied with—addition of a neutral mixture of sodium and potassium phosphates did not lead to increased growth. This is reflected in the composition of the blood and bones. Where no fat-soluble vitamins were added the calcium of the blood is consistently depressed with apparently the greatest depression where the most

phosphate was added. Radiation with ultra-violet light for 10 minutes daily in the absence of fat-soluble vitamins in the diet brought up both the calcium and phosphorus of the blood.

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## METABOLISM OF L-PYRROLIDONE CARBOXYLIC ACID AND ITS STABILITY TO ACIDS AND ALKALIES.\*

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In the study of the physiology of the amino-acids and their derivatives the cyclic compounds always engage attention because presumably the formation or disruption of ring structures requires special processes. No doubt there is considerable reason for assuming that such should be the case with homocyclic structures where there is union of carbon with carbon, but then evidence has been accumulated that the formation or oxidation of chain compounds is likewise not a simple matter.<sup>1</sup> Among the ring compounds considerable attention has been devoted to the metabolism of the pyrrole nucleus primarily through its participation in the structure of the pigments chlorophyll and hemoglobin as the alkylated pyrroles and in simple proteins where it occurs as proline, oxyproline, and forms part of the complex tryptophane and oxytryptophane nuclei.

Abderhalden has repeatedly called attention to the possibility of proline being a limiting factor in the growth processes of animals when not present in the amino-acid mixture of the proteins ingested. He has attempted to determine if the addition of proline to a proline-free ration would be able to restore growth in animals in which growth had been inhibited by feeding amino-acid mixtures as the source of protein, but invariably has met with failure. Apparently, part of the difficulty is due to the fact that proline is very easily decomposed, for even concentrat-

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<sup>1</sup> Abderhalden (1), p. 204.

ing proline solutions *in vacuo* causes them to acquire a pyridine-like odor.<sup>2</sup> Feeding of such proline soon causes anorexia.

Abderhalden<sup>2</sup> has also frequently suggested that possibly the animal possesses the ability to utilize pyrrolidone carboxylic acid in lieu of proline or glutamic acid as pyrrolidone carboxylic acid, like proline contains the pyrrole nucleus and is readily formed from glutamic acid.

Sure (2), working in this laboratory, has carried out a number of feeding trials using pyrrolidone carboxylic acid in lieu of proline, preliminary to work with proline, in an attempt to ascertain if the pyrrole nucleus must be present in the diet in other forms than in tryptophane.

As far as the writers are aware Abderhalden and Hanslian (3) are the only investigators who have attempted to determine the physiological properties and especially the lability of pyrrolidone carboxylic acid in the animal body. They did not find it entirely indifferent pharmacologically. In rabbits, 14 and 25 gm. given *per os* caused colitis followed by death, and in man, diarrhea was induced. Catabolism of the acid depended in part upon its optical activity. Some data suggested that the optically active form was metabolized whether introduced *per os* or subcutaneously, but the results were not decisive. When the racemic form was given, the *d* form appeared in the urine, indicating that the *l* form was more readily metabolized. Of 28 gm. of the racemic form, given subcutaneously to a rabbit in five doses, only part was absorbed before death ensued during the following night. From the urine in the bladder there were isolated 347 mg., mostly of the *d* form of the acid. In man, however, the *d* form seemed to be also readily metabolizable.

#### EXPERIMENTAL.

In the present series of experiments attempts were made to determine to what extent the animal is able to metabolize pyrrolidone carboxylic acid.

The acid was prepared from glutamic acid hydrochloride, isolated from the acid cleavage products of wheat gluten. From the hydrochloride, glutamic acid was made by adding an amount

<sup>2</sup> Abderhalden (1), p 213.

of sodium hydroxide in water solution exactly equivalent to its chlorine content as determined gravimetrically. The free acid was filtered off on a Buchner and washed with 60 per cent alcohol till free from chlorides. To convert it to pyrrolidone carboxylic acid, the method of Foreman was used (4). The glutamic acid was dissolved in water and boiled under a reflux condenser for 8 hours. Upon concentration to a syrup, and cooling, the residue solidified. The solidified mass was triturated thoroughly in a mortar with cold glacial acetic acid and filtered. The filtrate was concentrated on a water bath to a thick syrup and then crystallized in a desiccator over solid potassium hydroxide. After a few days the crystals of pyrrolidone carboxylic acid were filtered off by suction and finally dried *in vacuo* at room temperature until the odor of acetic acid could no longer be detected.

The purity of the resulting product was determined by making an amino nitrogen determination by means of the Van Slyke method, and a total nitrogen determination by the Kjeldahl method.

2 gm of the pyrrolidone carboxylic acid were dissolved in water and the volume was made up to 50 cc. 5 cc. aliquots were used for the amino nitrogen determination with the following results.

No	Nitrogen	Temperature	Pressure	Blank	Amino nitrogen.	Total nitrogen in amino form
	cc	°C	mm	cc	per cent	per cent
1	0.6	24.5	746	0.25	0.095	0.909
2	0.6	24.5	746	0.25	0.095	0.909

Two 0.4 gm. portions for nitrogen by Kjeldahl gave values of 10.42 and 10.45 per cent—theoretical 10.88. Examination in a polarimeter gave the value  $[\alpha]_D^{20} = -8.54^\circ$ . Foreman (4) has reported the value of  $-11.35^\circ$  and Abderhalden and Kautzsch (5)  $-10.8^\circ$  and  $-11.52^\circ$ . Evidently, the product obtained was partly racemized.

Two male pigs were used as the experimental animals. One weighed 31 kilos and the other 36 kilos. They were confined in metabolism cages for the quantitative collection of urine, and fed from 57 to 66 calories per kilo of body weight in the form of starch paste with distilled water *ad libitum*. After the nitrogen



excretion of the animal had reached a fairly constant level the diet was supplemented with varying amounts of nitrogen as pyrrolidone carboxylic and glutamic acids, fed in solution as the sodium salts. The dietary addition in each case was made by means of a stomach tube. Quantitative collection of urine was started after the 10th day of starch feeding. By that time the daily N excretion is usually fairly constant, being reduced to that originating from strictly endogenous sources. No harmful effects of prolonged confinement to a starch diet were noticed in either one of the two animals.

The urine was collected in a vessel in 24 hour intervals, preserved with toluene, and analyzed daily for total nitrogen, ammonia, urea, and amino nitrogen before and after hydrolysis.

Total nitrogen was determined on 10 cc. samples by the Kjeldahl method. Urea was determined on 5 cc. of urine, using the Benedict and Gephart (6) method. The ammonia was determined by the Folin (7) aspiration method, using a 25 cc. sample.

Amino nitrogen before and after hydrolysis was determined on 5 cc. of urine in the Van Slyke apparatus. Hydrolysis of the urine was carried out as follows 5 cc. of concentrated hydrochloric acid were added to 25 cc. of urine in a 200 cc. Erlenmeyer flask and the solution was boiled down to approximately 10 cc. The contents of the flask were then boiled for 2 hours under a reflux, 20 cc. of 10 per cent sodium hydroxide were added, followed by 5 cc. of acetic acid, and the solution was made up to a volume of 50 cc. Aliquots of 10 cc., corresponding to 5 cc. of urine, were used for the determination of amino nitrogen.

Table I presents the data obtained with Pig I. With the administration of 0.9679 gm. of pyrrolidone carboxylic acid no change in total nitrogen, urea, ammonia, or in amino nitrogen after hydrolysis was observed. The latter value should have increased if the acid were excreted in part in the unchanged form because by boiling with acid, glutamic acid is regenerated from pyrrolidone carboxylic acid. Increase in amino nitrogen without hydrolysis would have indicated a transformation of the acid to glutamic acid with failure of metabolism of the latter. This also was not the case. Apparently, the nitrogen of the pyrrolidone carboxylic acid was all retained.

Increasing the ingestion of pyrrolidone carboxylic acid to 2.154 gm. of nitrogen did not yield any very definite results.

TABLE I.

Fig I. Male. Weight 31 kilos. Caloric intake as starch, 66 cal. per kilo.

Day of starch feeding	Total nitrogen	Ammonia nitrogen	Urea nitrogen	Amino nitrogen	Amino N after hydrolysis	Dietary additions.
	gm	gm	gm	gm	gm	
10	2 720	0 277	1.973		0.652	
11	2 960	0 286	2 121	0 455	0 631	
12	2 850	0 252	2 018	0 446	0.606	0.9679 gm. N as pyrrolidone carboxylic acid.
13	2 505	0 246	1 794	0 327	0 444	
14	3 160	0 272	2 338	0 507	0 585	
15	1 964	0 176	1 472	0.305	0 349	
22	2 184	0 221	1 514	0 296	0 400	
23	2 702	0 264	1 934	0 529	0 575	
24	1 800	0 166	1 290	0 354	0 367	2 154 gm N as pyrrolidone carboxylic acid.
25	2 883	0 243	1 815	0 435	0 459	
26	2 319	0 276	1 836	0 360	0 443	
27	2 328	0 151	1 539	0 431	0 457	
30	2 031	0 229	1 320	0 385	0 399	
31	1 929	0 213	1 195	0 371	0 392	
32	3 442	0 237	2 692	0 430	0 446	2 gm N as urea
33	2 368	0 255	1 560	0 469	0 471	
34	2 158	0 354	1 353	0 374	0 374	
35	1 877	0 324	1 137	0 340	0 400	
36	2 352	0 152	1 810	0 371	0 376	2 gm N as glutamic acid.
37	3 246	0 166	2 148	0 946	1 004	
38	1 900	0 154	1 214	0 290	0 421	
39	1 989	0 233	1 215	0 390	0 454	
40	2 256	0 312	1 401	0 294	0 320	
41	1 829	0 324	1 104	0 299	0 299	
42	2 184	0 396	1 186	0 388	0 404	
43	1 884	0 363	1 067	0 272	0 304	
44	3 515	0 342	1 317	0 271	1 830	4.196 gm N as pyrrolidone carboxylic acid.
45	3 058	0 179	2 071	0 303	0 442	
46	2 463	0 152	1 599	0 252	0 430	
47	1 912	0 227	1 146	0 279	0 384	

If an average is taken of a 2 day period representing the 2 days following the administration of the acid for comparison with an average of 2 days before and 1 day after, about 30 per cent of

the nitrogen was excreted. But isolated previous values are almost as large as the maximum of this period, making conclusions problematical. An increase in amino nitrogen is also questionable.

The above data made it desirable to have information on the excretion of other nitrogenous compounds, *viz.* urea and glutamic acid, the former as an end-product of the metabolism and the latter as an intermediary compound. It was possible that the pig confined to an endogenous plane of protein metabolism for a prolonged period might have its tissues so depleted in soluble nitrogen compounds that small amounts of nitrogen administered irrespective of kind might be generally retained.

To determine this, urea and glutamic acid were given to the pig. Table I shows that administered urea N was readily excreted. Taking 2 days before and 2 days after the administration, 80 per cent of the urea nitrogen made its appearance in the urine within 48 hours, in fact, 72 per cent was excreted in the first 24 hours—and this all in the form of urea. Glutamic acid obeys different laws. Using the same method of calculation, 78 per cent of the nitrogen was excreted in the 48 hours following, but only 17 per cent—instead of the 72 per cent in the case of urea—was excreted in the first 24 hours. Not all the increase of nitrogen was accounted for by the urea, practically 0.5 gm. of the nitrogen was excreted as amino nitrogen, showing that glutamic acid is not readily metabolized by the pig. This nitrogen was excreted in the second 24 hours of the period.

After these trials, showing that nitrogen in small amounts can be traced into the urine, pyrrolidone carboxylic acid was again given to the pig, this time to the extent of 4.196 gm. of nitrogen. Taking the average of 3 days preceding this period as a normal, 74 per cent of the ingested nitrogen was excreted in the following 3 days, but only a 38 per cent increase was shown in the urea excretion. The balance was accounted for by increased amino nitrogen after hydrolysis which totalled 38 per cent. There resulted no increase in free amino nitrogen, showing that when pyrrolidone carboxylic acid is administered part of it escapes metabolism as such, rather than as glutamic acid into which it is convertible. The data show clearly that of the 4.196 gm. of pyrrolidone carboxylic acid nitrogen given, only three-fourths

made its appearance in the urine, of which one-half represented the unchanged acid.

That this actually represented unchanged pyrrolidone carboxylic acid was proved by its isolation. The urine left over from the quantitative analyses was evaporated to a thick syrup and then allowed to crystallize. The hard crystalline mass was triturated in a mortar with cold glacial acetic acid and filtered. The acid solution was then set aside in a desiccator over solid potassium hydroxide to crystallize. There were finally obtained 1.89 gm. of crystals, dried *in vacuo*, containing only 0.09 per cent

TABLE II

Pig II. Male. Weight 36 kilos Caloric intake as starch, 57 cal per kilo.

Day of starch feeding	Total nitrogen	Ammonia nitrogen	Urea nitrogen	Amino nitrogen	Amino N after hydrolysis	Dietary additions
	gm	gm	gm	gm	gm	
11	2 678	0 392	1 562	0 463	0 520	4 gm. N as pyrrolidone carboxylic acid.
12	2 304	0 343	1 342	0 343	0 430	
13	2 472	0 210	0 960	0 245	1 058	
14	4 167	0 120	3 032	0 447	0 667	
15	2 746	0 081	2 100	0 192	0 379	
16	1 967	0 064	1 255	0 182	0 396	4 gm. N as pyrrolidone carboxylic acid.
17	1 954	0 135	1 181	0 257	0 373	
18	1 833	0 202	1 016	0 251	0 422	
19	2 838	0 281	0 966	0 349	1 349	
20	3 252	0 125	2 425	0 452	0 555	
21	2 762	0 087	2 082	0 354	0 412	
22	2 313	0 070	1 690	0 302	0 346	

amino nitrogen and 10.46 per cent total nitrogen; theoretical 10.18 per cent N. Examination in a polarimeter revealed a specific rotation of  $-8.3^\circ$ , the same as before, indicating no differential metabolism between the *d* and *l* forms.

In Table II are presented data from Pig II. This pig was likewise given 4 gm. of N as pyrrolidone carboxylic acid, at different times. In both cases only partial metabolism of the acid resulted as shown by the pronounced increase of amino nitrogen after hydrolysis. In the first case there was an increase of 14 per cent and in the latter case 23 per cent on the 1st day after administration. The following day this was reduced to 4 per cent in both cases. Increase in amino nitrogen was slight

and questionable. The data on total nitrogen are not clean cut, due to irregularity in output. Practically all that can be said, is that there was a pronounced increase in excretion of total N. Urea excretion is decreased on the 1st day with a large increase later. The cause of this initial decrease is not clear. There was no acidosis, the ammonia nitrogen in fact being decreased decidedly by the influence of the alkali liberated from the oxidized sodium salt. It is all the more remarkable in view of the fact that unchanged pyrrolidone carboxylic acid was rapidly excreted in large amounts.

The resistance of pyrrolidone carboxylic acid to destruction in the animal body raised the question if such amounts as are metabolized may not be destroyed by virtue of the fact that they are hydrolyzed to glutamic acid by the digestive secretions and that thus the way is laid open for deaminative and oxidative processes.

The effect of acids on the equilibrium, pyrrolidone carboxylic acid  $\rightleftharpoons$  glutamic acid, has been studied in brief by Foreman (4), and Abderhalden and Kautzsch (5). Foreman showed that glutamic acid was readily converted into pyrrolidone carboxylic acid by boiling its aqueous solution for 5 hours under a reflux condenser. Over 50 per cent was converted by this process. Aspartic and acetic acids had no effect on this reaction, but hydrochloric acid and sulfuric acid of 1 to 2 per cent concentration reduced the conversion to less than one-third. Foreman concludes that about 3 per cent hydrochloric acid and 8 per cent sulfuric acid would prevent the reaction entirely. The reverse reaction, that is, the conversion of pyrrolidone carboxylic acid to glutamic acid, was accomplished readily with slight discoloration by boiling with strong HCl. Abderhalden and Kautzsch found that 0.5 gm., dissolved in water and saturated with HCl upon evaporation to dryness, gave glutamic acid hydrochloride. The influence of concentration of acid and time on the conversion were not determined. All in all, the data are too limited to allow correlation with our animal experiments. Before proceeding with final determinations a preliminary test of the convertibility of pyrrolidone carboxylic acid to glutamic acid was carried out. 2 gm. of pyrrolidone carboxylic acid were dissolved in 100 cc. of 5 per cent hydrochloric acid and boiled under a

TABLE III

*Conversion of Pyrrolidone Carboxylic Acid to Glutamic Acid by Boiling with 1.0 Per Cent Hydrochloric Acid.*

Period of hydrolysis	Nitrogen	Temperature	Pressure	Blank	Amino nitrogen	Total nitrogen in amino form.
hrs.	cc	°C	mm	cc	per cent	per cent
1	9.9	22.5	740	0.25	5.196	49.72
2	12.8	25.5	740	0.25	6.758	64.67
3	14.0	25.5	740	0.25	7.404	70.85
5	15.1	25.5	740	0.25	7.996	76.52
7	15.2	25.5	740	0.25	8.104	77.52
24	15.4	24.0	742	0.25	8.226	78.72

TABLE IV.

*Conversion of Pyrrolidone Carboxylic Acid to Glutamic Acid by Boiling with 0.62 Per Cent (N/7) Hydrochloric Acid.*

Period of hydrolysis	Nitrogen	Temperature	Pressure	Blank	Amino nitrogen	Total nitrogen in amino form
hrs	cc	°C	mm	cc	per cent	per cent
1	5.65	23.5	737	0.25	2.926	28.00
2	7.20	23.5	737	0.25	4.185	40.04
3	8.95	23.5	737	0.25	4.850	46.32
5	10.90	25.0	737	0.25	5.726	54.79
8	11.60	22.0	740	0.25	6.225	59.57
12	13.00	22.0	740	0.25	6.990	66.92
24	14.25	24.0	740	0.25	7.602	72.74

TABLE V

*Conversion of Pyrrolidone Carboxylic Acid to Glutamic Acid by the Action of 1 Per Cent Hydrochloric Acid at 37.5°C.*

Period of hydrolysis	Nitrogen	Temperature	Pressure	Blank	Amino nitrogen	Total nitrogen in amino form
days	cc	°C	mm	cc	per cent	per cent
1	0.8	24.5	744	0.25	0.2994	2.86
2	1.5	24.5	744	0.25	0.6806	6.51
3	2.25	25.0	745	0.25	1.0874	10.40
4	2.90	24.0	740	0.25	1.4380	13.76
5	3.25	22.0	741	0.25	1.6470	15.76
6	3.95	24.5	739	0.25	1.9437	18.60
7	4.50	24.0	734	0.25	2.2865	21.88
8	4.80	24.0	734	0.25	2.4479	23.42

reflux condenser. The amino nitrogen was determined in 5 cc. portions after 1 hour hydrolysis and at the end of 24 hours with the following result.

Period of hydrolysis	Nitrogen	Temperature	Pressure	Blank	Amino nitrogen	Total nitrogen in amino form
<i>hrs</i>	<i>cc.</i>	<i>°C</i>	<i>mm</i>	<i>cc.</i>	<i>per cent</i>	<i>per cent</i>
1	18.9	22	745	0.25	10.31	98.82
24	19.4	23	744	0.25	10.41	99.69

It is evident that a comparatively low concentration of acid is able to effect the conversion. Definite results, varying the time, temperature, and concentration of acid, are shown in Tables

TABLE VI

*Conversion of Pyrrolidone Carboxylic Acid to Glutamic Acid by the Action of 0.52 Per Cent (N/7) Hydrochloric Acid at 37.5°C*

Period of hydrolysis	Nitrogen	Temperature	Pressure	Blank	Amino nitrogen	Total nitrogen in amino form
<i>days</i>	<i>cc</i>	<i>°C</i>	<i>mm</i>	<i>cc</i>	<i>per cent</i>	<i>per cent</i>
1	0.6	24.5	744	0.25	0.1905	1.82
2	1.0	24.5	744	0.25	0.4083	3.90
3	1.40	25.0	745	0.25	0.6252	5.98
4	1.75	24.0	740	0.25	0.8145	7.79
5	1.95	22.0	741	0.25	0.9336	8.93
6	2.10	24.5	739	0.25	1.0002	9.57
7	2.30	24.0	734	0.25	1.1029	10.55
8	2.30	24.0	734	0.25	1.2374	11.84

III, IV, V, and VI. The values for amino nitrogen were obtained by dissolving 2 gm. of pyrrolidone carboxylic acid in 100 cc. of acid and removing 5 cc. aliquots at definite intervals for the amino nitrogen determinations.

The data on the action of acid show that with a maximum concentration of 0.5 per cent hydrochloric, at approximately body temperature, less than 2 per cent of the pyrrolidone carboxylic acid is converted to glutamic acid in 24 hours, less than 4 per cent in 48 hours, and less than 8 per cent in 96 hours. As the pyrrolidone carboxylic acid nitrogen when given to a pig in one case was excreted as urea nitrogen to the extent of 38 per cent of the amount ingested, it is obvious that gastric acidity cannot be responsible for its cleavage.

Our data also show that alkali likewise cannot be an important factor in the *intra vitam* hydrolysis of the acid. 1 gm. of pyrrolidone carboxylic acid was dissolved in 200 cc. of 0.5 per cent sodium carbonate solution and another 1 gm. portion in 100 cc. of 5.0 per cent sodium carbonate. Both were boiled under a reflux condenser, and 5 cc. aliquots were removed for amino nitrogen determination from time to time. The results are shown in Table VII.

TABLE VII

*Conversion of Pyrrolidone Carboxylic Acid to Glutamic Acid by Boiling with, Respectively, 0.5 and 5.0 Per Cent Sodium Carbonate Solutions.*

Strength of sodium carbonate	Period of hydrolysis	Nitrogen	Temperature	Pressure	Blank	Amino nitrogen.	Total nitrogen in amino form
per cent	hrs	cc	°C	mm	cc	per cent	per cent
0.5	1	0.5	22.5	744	0.25	0.55	5.26
0.5	24	1.6	23.0	744	0.25	2.96	28.32
5.0	1	1.8	24.5	734	0.25	1.66	15.88
5.0	24	7.5	24.0	744	0.25	7.91	75.70
5.0	48	9.4	23.5	738	0.25	9.93	95.02

## SUMMARY.

Pyrrolidone carboxylic acid can be metabolized by the animal organism. When ingested in large amounts, it is partly excreted unchanged in the urine. When incompletely metabolized no increase in amino nitrogen of the urine results, showing that hydrolysis does not proceed so rapidly as to lead to the production of amounts of glutamic acid in excess of the animal's ability to deaminize it completely. The acidity or alkalinity of the digestive tract is not sufficient to account for the hydrolysis of the pyrrolidone carboxylic acid. Its cleavage as well as its deamination must therefore be a function of body tissue.

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## PROTEINS OF WHEAT BRAN.

### I. ISOLATION AND ELEMENTARY ANALYSES OF A GLOBULIN, ALBUMIN; AND PROLAMINE.\*

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Wheat has been from time immemorial and continues to be probably the most important item in the food of civilized man. The proteins of the wheat germ, and those proteins of the endosperm which collectively are commonly known as gluten, have been extensively investigated by Osborne and coworkers and others. We have but little knowledge, however, of the proteins contained in the milling by-product of wheat commonly called bran.<sup>1</sup>

On account of the thick walls of the cells in the layer containing the proteins, the extraction of the proteins from the bran has been considered so difficult that they have never been isolated in sufficient quantity or suitable condition for a study of their chemical or physical character. Furthermore, even the cleanest grades of commercial wheat bran always contain an admixture of an appreciable quantity of starch and gluten, which are removed with difficulty.

Bran is extensively used in the feeding of farm animals and has been long regarded as having a high nutritive value. During recent years there has been an increasing recognition of the

\* A preliminary report of this paper was presented at the meeting of the American Chemical Society held in New Haven, Conn., April 2 to 9, 1923.

<sup>1</sup> The term "bran" is used in this paper to include the outer seed coats, together with the underlying layer of cells which contains the protein, and which in the milling process is removed with, and is firmly attached to, the outer seed coats.

value of bran as an element in human food. Commercial methods are now perfected for the preparation of bran as a palatable cereal breakfast food.

It is generally known that the nutritive value of so called "patent" wheat flour is inferior to that of whole wheat, being deficient in vitamins, inorganic constituents, and defective in the quality of its proteins. It therefore appears very probable that the proteins of the bran are of a different chemical nature, and contain satisfactory quantities of those amino-acids which are deficient in the gluten proteins. Although the results of elementary analyses of the proteins reported in this paper support the above supposition, we do not wish to draw any final conclusions as to the identity or non-identity of the bran proteins with the corresponding proteins of the other parts of the wheat kernel, until we have obtained further knowledge of the amino-acid composition of the bran proteins. Experiments to this end are now in progress.

In their classic work on "The proteins of the wheat kernel." Osborne and Voorhees<sup>2</sup> have shown that the proteins of wheat gluten, which comprises the proteins of white, wheat flour, consist chiefly and in about equal quantities of gliadin, the alcohol-soluble protein, and glutenin, a protein which is insoluble in water, neutral salt solutions, or alcohol, but is soluble in dilute acids or alkalis. The proteins of the wheat embryo as shown by Osborne and Campbell<sup>3</sup> differ distinctly in character from those of the gluten, and consist of globulin, albumin, and proteose. These proteins, however, constitute but a relatively small proportion of the total protein of the wheat kernel.


The composition of bran varies with the kind of wheat and the locality in which it is grown. The following composition of a sample of commercial bran is given by Osborne and Mendel.<sup>4</sup>

*Composition of Commercial Bran, Moisture-Free*

	<i>per cent</i>
Sucrose . . . . .	1 64
Dextrin . . . . .	4 19
Starch . . . . .	13 39

<sup>2</sup> Osborne, T. B., and Voorhees, C. G., *Am. Chem. J.*, 1893, xv, 392.

<sup>3</sup> Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1900, xxii, 379.

<sup>4</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557. 

Pentosans, etc .....	27 58
Protein ( $N \times 6.25$ ) .. .	17 00
Fiber.....	9.37
Fat .. .	7 07
Ash .....	7 24
Undetermined .....	12.52
	<u>100 00</u>

Bran also contains relatively large quantities of inorganic salts. Jordan, Hart, and Patten<sup>5</sup> give the following analyses.

$P = 1.42$ ,  $Ca$  as  $CaO = 0.18$ ,  $Mg$  as  $MgO = 0.89$ ,  $K$  as  $K_2O = 1.58$

The approximate proportion of endosperm, bran, and embryo in the wheat kernel, the proportion of the total protein of the seed in these parts and also the percentage of the total protein in the part itself, is given in the accompanying tabulation by Osborne and Mendel.<sup>4</sup>

	Part as per cent of seed	Per cent of seed as nitrogen in part	Per cent of seed as protein in part ( $N \times 5.7$ )	Per cent of protein in part	Per cent of total protein of seed in part
Endosperm	83.5	1.61	9.17	11.1	73.3
Bran . . . .	15.0	0.49	2.80	18.7	22.3
Embryo . . . .	1.5	0.10	0.57	36.7	4.4
	<u>100.0</u>	<u>2.20</u>	<u>12.54</u>		<u>100.0</u>

In view of the little that is known regarding the proteins of wheat bran, its relatively high content of protein, and of its nutritional and economic importance, we have undertaken an investigation of the proteins of wheat bran with the view of their isolation and the determination of their physical and chemical characters, and of obtaining such information concerning the individual proteins of bran as we already have regarding the proteins of the other parts of the wheat kernel.

In this paper are described the preparation and properties of three proteins which have been isolated from wheat bran so treated as to remove most of the other parts of the seed which

<sup>5</sup> Jordan, W. H., Hart, E. B., and Patten, A. J., *Am. J. Physiol.*, 1906, xvi, 268.

are usually found associated with commercial bran. The proteins isolated consisted of an albumin, a globulin, and a prolamine.

The isolation and preparation of proteins to be used for a study of their properties and composition require that they be obtained in as pure a state as possible. This calls for methods of purification, such as reprecipitations, repeated washings, and filtration—processes which frequently involve large losses of material. Furthermore, in many cases the extractions of the

TABLE I

*Proteins Extracted and Isolated from Wheat Bran.*

Total protein in the bran ( $N \times 6.25 = 17.25$  per cent).

	Nitrogen extracted (per cent of bran)	Protein extracted $N \times 6.25$ (per cent of bran)	Protein isolated	
			Per cent of bran	Per cent of total protein in bran
Extracted by distilled water	0.63	3.94		
1. Albumin			2.87	16.64
2. Globulin			0.69	4.00
Extracted by 4 per cent sodium chloride	0.35	2.19		
Globulin			1.66	9.62
Extracted by 70 per cent alcohol	1.01	6.31		
Prolamine			5.35	31.01
Extracted by 0.5 per cent sodium hydroxide	0.40	2.50		
Total		14.94*	10.57	61.27

\* Equivalent to 86.61 per cent of the calculated total protein in the bran.

proteins from their parent substances have been incomplete, the object having been merely to secure sufficient quantities of the purified proteins for their chemical study. Consequently, the yields of the proteins as finally obtained oftentimes do not nearly represent the quantities in which they are present in substances from which they were obtained. To know as nearly as possible the percentages of the different proteins present in a given seed or foodstuff is frequently of great importance. In order to obtain as accurately as possible the percentages of the different proteins present in wheat bran, successive exhaustive extractions of

the washed bran meal were made with distilled water, 4 per cent aqueous sodium chloride solution, boiling 70 per cent alcohol, and with 0.5 per cent sodium hydroxide. The proteins removed by these various solvents were separated and isolated by methods involving minimum losses. The yields found are con-

TABLE II.  
*Average Results of Duplicate Analyses of Wheat Bran Albumin.\**

	Preparation.					
	I	II	III	IV	V	Average
	per cent	per cent	per cent	per cent	per cent	per cent
C	53 20	53 25	53 27	53 29	53 05	53 21
H	6 79	6 77	6 77	6 47	6 75	6 71
N	15 09	15 51	15 28	15 61	15 61	15 42
S	1 24	1 39	1 38	1 43	1 33	1 35
O	23 68	23 08	23 30	23 20	23 26	23 30
Moisture	10 24	5 08	3 95	4 40	4 66	
Ash	1 49	0 65	0 29	0 41	4 59	

\* Calculated on an ash- and moisture-free basis.

TABLE III  
*Average Results of Duplicate Analyses of Wheat Bran Globulin \**

	Preparation							
	I	II	III	IV	V	VI	VII	Average
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
C	53 41	53 49	53 58	53 37	53 59	53 41	53 19	53 43
H	7 68	7 69	7 37	7 35	7 30	7 34	7 10	7 40
N	17 94	17 89	17 81	18 01	17 67	17 39	17 59	17 76
S	0 91	0 92	0 90	0 92	0 89	0 90	0 90	0 91
O	20 06	20 01	20 34	20 35	20 55	20 96	21 22	20 50
Moisture	10 11	9 20	4 70	5 16	10 33	5 01	4 16	
Ash	0 60	1 25	0 24	0 36	1 49	1 81	0 17	

\* Calculated on an ash- and moisture-free basis.

siderably higher than those obtained in the preparation of samples for the analytical study of the proteins, and, we believe, quite closely represent the quantities in which these proteins were present in the bran.

By exhaustively extracting the bran with the solvents used, there was removed an amount of nitrogen equivalent to 86.61 per cent of the calculated ( $N \times 6.25$ ) total protein in the bran. From these extracts, there were isolated the following percentages of proteins, expressed in terms of the bran used: albumin, 2.87 per cent; globulin, 2.35 per cent; and prolamine, 5.35 per cent. The results of these extraction experiments are given in Table I.

Several preparations of each of the three proteins isolated from bran were made by different methods. It is of interest to note the close agreement of the results obtained on analyses

TABLE IV.  
*Average Results of Duplicate Analyses of the Alcohol-Soluble Protein from Wheat Bran \**

	Preparation					Average
	I	II	III	IV	V	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C . . . .	54 33	54 05	54 20	54 38	54 30	54 25
H .. . .	6 72	6 74	6 78	6 64	6 87	6 75
N .... .	15 23	15 56	15 34	15 42	15 21	15 35
S .. . .	1 36	1 26	1 37	1 37	1 38	1 35
O . . . .	22 36	22 39	22 31	22 19	22 24	22 30
Moisture	8 00	8 24	3 25	4 51	5 23	
Ash .. .	0 86	0 46	0 19	1 77	8 07	

\* Calculated on an ash- and moisture-free basis.

of the same protein obtained by different methods. (Tables II, III, and IV.) The composition of these proteins differs from that of similar proteins obtained by Osborne and Voorhees<sup>2</sup> from "shorts," a commercial product consisting largely of bran but also containing considerable portions of the starchy endosperm of the seed.

#### EXPERIMENTAL.

*Preparation of Material.*—The bran used for the experiments described in this paper was furnished by the Hecker-Jones-Jewell Milling Company, and was obtained mostly from 75 per cent Dark 1 Northern Spring Wheat and 25 per cent of 2 Hard Kansas Wheat. Previous to grinding, the wheat had been tem-

pered by the addition of water and passed to bins at room temperature, where it remained for 8 hours before grinding. Addition of moisture before milling is made to toughen the bran so as to facilitate its removal from the other parts of the kernel. Commercial bran always contains appreciable quantities of adhering parts of the rest of the kernel. In order to remove as much as possible of this adhering material, the bran was placed in cheese-cloth and rapidly washed by vigorous plunging in cold tap water. By this method most of the adhering starchy endosperm was removed. The turbid washings were reserved for later examination. Immediately after washing, the bran was dried at 30°C. in a motor driven fan drier, and ground so as to pass through a 100 mesh sieve. The washing and drying caused the bran to lose much of its tough character, making it possible to grind it more easily. Preliminary extractions of the bran with protein solvents showed, however, that equally efficient extractions of the protein could be obtained from bran which had been ground to pass through a 40 mesh sieve.

Microscopical examinations of both the unwashed and washed bran were made by J. F. Clevenger, of the Pharmacognosy Laboratory of this Bureau. It was estimated that at least 75 per cent of the starchy endosperm in the original bran had been removed by the washing, and that the resulting washed and dried bran contained less than 5 per cent of material other than bran. The presence of embryonic tissue in the original bran could not be detected. Analysis of the washed bran showed a content of 3.6 per cent of starch. This percentage is in fair agreement with the estimate based on the microscopical examination. Since the ratio of protein to starch in wheat endosperm is approximately 1:7, as pointed out by Osborne and Mendel,<sup>4</sup> the amount of endosperm protein in our washed bran can be estimated from its starch content at approximately 0.5 per cent. It is not believed, however, that this small quantity of protein derived from the starchy endosperm is sufficient to cause any appreciable error in the data presented in this paper on the proteins of the bran.

Total nitrogen determinations, calculated on an ash- and moisture-free basis, showed that the unwashed bran contained 2.84 per cent of nitrogen, or 17.75 per cent of protein ( $N \times 6.25$ ),



*Extraction with 4 Per Cent Sodium Chloride Solution.*—The residue remaining after the extractions with distilled water was next extracted with 4 per cent sodium chloride solution at room temperature. Nitrogen determinations made on aliquot portions of the extracts showed that exhaustive extraction with the salt solution removed 0.35 per cent of nitrogen, equivalent to 2.19 per cent of protein. The joint saline extracts were acidified with acetic acid, and boiled until there was no further separation of coagulum. The precipitate was collected on a filter, washed with distilled water, and dried at 110°. The yield of globulin thus obtained was 1.66 per cent of the meal, or 9.62 per cent of the total protein in the bran.

*Extraction with 70 Per Cent Alcohol.*—The residue remaining after the extraction with sodium chloride solution was exhaustively extracted with boiling 70 per cent alcohol. The alcohol removed 1.01 per cent of nitrogen, equivalent to 6.31 per cent of protein. The joint extracts were filtered and concentrated under reduced pressure until most of the alcohol was removed. The flocculent precipitate, which separated as the alcohol was being removed, was allowed to settle and was washed by decantation with water and dried at 110°C. It amounted to 5.35 per cent of the meal, or 31.01 per cent of the total protein in the meal based on  $N \times 6.25$ .

*Extraction with 0.5 Per Cent Sodium Hydroxide Solution.*—The residue from the alcohol extractions was then extracted with 0.5 per cent sodium hydroxide at room temperature. The alkaline extracts contained 0.40 per cent of nitrogen, equivalent to 2.50 per cent of protein. The joint extracts were acidified with acetic acid, and several volumes of alcohol were added, which caused the precipitation of a substance containing but a small amount of nitrogen.

Qualitative tests made on this substance for phytin and purines gave negative results, neither did it respond to the Biuret, glyoxylic, or Millon tests for protein. The material ignited readily, leaving a large ash residue.

Exhaustive extractions with these four solvents therefore removed nitrogen equivalent to 14.94 per cent of protein, calculated on the basis of the bran used. This is equivalent to 86.61 per cent of the calculated total protein in the bran. There

were actually isolated as identified proteins, 2.35 per cent of globulin, 2.87 per cent of albumin, and 5.35 per cent of prolamine—a total of 10.57 per cent of the bran or 61.27 per cent of the calculated total protein in the bran. The results of these exhaustive extractions are summarized in Table I.

Since probably not all the nitrogen in bran is protein nitrogen, the actual quantity of protein present would therefore be less than the 17.25 per cent obtained by multiplying the nitrogen by 6.25. This would give a correspondingly higher value for the percentage of protein actually isolated than the 61.27 per cent here reported.

### *Preparation of the Proteins.*

All the preparations described and analyzed, excepting one, were obtained by successive extractions of each sample of the washed bran meal by the various solvents.

*The Albumin.*—The albumin preparations were obtained by the following methods.

1. A distilled water extract of the bran was dialyzed for 10 days against chilled running water and the dialysate saturated with washed carbon dioxide. The small quantity of precipitated globulin was removed by filtration, and the filtrate was heated for 30 minutes at 65°C. The coagulated protein was thoroughly washed with distilled water and dried in the usual way with alcohol and ether. The yield was 0.1 per cent (Preparation I).

2. Preparation II was obtained in a 0.26 per cent yield by dialysis of a 4 per cent sodium chloride extract against chilled running water for 12 days, and subsequent treatment of the dialysate as given for Preparation I.

3. A 4 per cent sodium chloride extract was saturated with ammonium sulfate and the precipitated protein redissolved by addition of distilled water. The solution was filtered and dialyzed for 16 days. After removal of the globulin, the dialysate was then treated as in the two previous preparations (Preparation III). A yield of 0.18 per cent was obtained.

4. A distilled water extract without previous dialysis was saturated with washed carbon dioxide, and then slightly acidified with 1 per cent acetic acid. The precipitated globulin was re-

moved by filtration, and the albumin coagulated by heating the filtrate for 30 minutes at 65°C. The albumin, after washing and drying in the usual way, was obtained in 0.99 per cent yield (Preparation IV).

5. A 4 per cent sodium chloride extract of the bran was saturated with washed carbon dioxide gas. This caused but an incomplete precipitation of the globulin. The remainder was separated by slowly adding, drop by drop, dilute acetic acid, the end-point being indicated by the flocculent character of the precipitated protein. After removal of the globulin by filtration, the albumin (Preparation V) was coagulated, washed, and dried, as in the case of the previously described preparations (yield, 2 per cent).

The average results of duplicate analyses of these five albumin preparations, calculated on an ash- and moisture-free basis, are given in Table II.

*The Globulin.*—The globulin preparations were obtained from a 4 per cent aqueous sodium chloride extract of the bran meal. Seven preparations were made by various methods. In all cases the precipitated proteins were finally washed and dried in the usual way.

1. Preparation I was obtained in 0.63 per cent yield by making the extract 0.55 saturated with ammonium sulfate. The precipitate was removed by filtration, redissolved by addition of water, and the solution was filtered. The globulin was then precipitated from the clear filtrate by dialysis.

2. Preparation II was made in the same way as Preparation I, with the exception that the extract was made 0.65 saturated with ammonium sulfate. Yield, 1.0 per cent.

3. By complete saturation of the extract with ammonium sulfate, and subsequent removal of the globulin by dialysis, Preparations III and IV were obtained in 0.97 and 0.83 per cent yields.

4. Preparations V and VI were obtained by directly dialyzing the salt extract against chilled running water for 12 days.

5. Acidification of the extract with carbon dioxide, followed by the addition of dilute acetic acid, as described under the preparations of the albumin (Method 5), yielded Preparation VII in 2 per cent yield. Average results of the analyses of these preparations are given in Table III.

*The Alcohol-Soluble Protein.*—Five preparations of the alcohol-soluble protein, made in several ways, were obtained as follows: Preparations I to IV, inclusive, were isolated from bran meal which had been previously exhaustively extracted with distilled water and 4 per cent sodium chloride solution. This residue was extracted with boiling 70 per cent alcohol for 15 minutes, and the hot mixture was immediately filtered. Preparation I was obtained by pouring the extract into a 4 per cent sodium chloride solution. The precipitate was thoroughly washed with water and dried in the usual way. The yield was 1.0 per cent of the original bran used before the extraction with water and salt.

Concentration of the alcoholic extract under diminished pressure until most of the alcohol had been removed caused the separation of the prolamine in a form which was no longer soluble in alcohol. This precipitate constituted Preparation II. The yield was 1.7 per cent.

Preparation III was isolated from a highly concentrated alcoholic solution of the protein. This solution was obtained by occasional addition during the distillation under diminished pressure of small quantities of 95 per cent alcohol, so as to prevent precipitation of the protein due to the diminishing content of alcohol in the distillation reservoir. The protein was precipitated from the concentrated solution by addition of several volumes of absolute alcohol. After washing and drying the precipitated protein in the usual way, a yield of 4.29 per cent was obtained.

Preparation IV, obtained in 1 per cent yield, was isolated as in the case of Preparation III, with the exception that the product obtained by precipitation with absolute alcohol was redissolved in boiling 80 per cent alcohol and then reprecipitated by absolute alcohol, after having first filtered off some undissolved protein which had become denatured.

Preparation V was isolated from bran which had not been previously extracted with other solvents. The method of preparation otherwise was the same as that used for Preparation III. Because of an accidental loss of a portion of the extract used in this preparation only a small yield was obtained.

The elementary composition of the prolamine preparations is given in Table IV.

*Properties of the Proteins.*

*The Albumin.*—The albumin preparations obtained as already described ranged in color from pale cream to gray.

In aqueous solution, slightly acidified with acetic acid, the albumin coagulated at 60°C. When heated in 4 per cent aqueous sodium chloride solution, it coagulated at 65°C., and at 62°C. in a 4 per cent sodium sulfite solution. Its coagulation point in water is somewhat raised by the presence of a small quantity of the globulin.

*The Globulin.*—Dilution of saline extracts of the bran with large volumes of water showed that the globulin was readily soluble in dilute aqueous sodium chloride solutions. Consequently, its preparation by the customary method of diluting the saline extract is impracticable. It can be precipitated in small quantities from a sodium chloride solution by saturation with carbon dioxide, and practically completely by slight acidification with acetic acid. The precipitate thus formed is readily soluble in 4 per cent sodium chloride solution.

The precipitation limits with ammonium sulfate ranged from 0.4 to 0.65 of saturation.

The globulin coagulates in a slightly acidified 4 per cent sodium chloride solution at 95°C.

The preparations were obtained in the form of dusty powders, ranging in color from white to cream.

*The Alcohol-Soluble Protein.*—This protein precipitated by absolute alcohol from a concentrated alcoholic solution is readily soluble in boiling 80 per cent alcohol. It differs from *gliadin* in its behavior when separating on cooling from warm 70 per cent alcohol. Much of this protein rapidly precipitates in a finely divided form, which redissolves on warming. Addition of aqueous sodium chloride to its alcoholic solution causes the prolamine to separate in flocculent particles which soon adhere to form a sticky precipitate. Addition of 2 volumes of 2 per cent hydrochloric acid to the prolamine in 70 per cent alcohol produces a flocculent precipitate which redissolves on boiling and again separates on cooling.

## SUMMARY.

Three proteins, an albumin, a globulin, and an alcohol-soluble protein, have been isolated from wheat bran. The bran, which originally was exceptionally clean, was rapidly washed in cold water and immediately dried at a low temperature. In this way the greater part of the adhering particles of other portions of the wheat kernel was removed. The bran was then ground to a fine powder. This contained 17.25 per cent of protein ( $N \times 6.25$ ). By successive exhaustive extractions with distilled water, 4 per cent sodium chloride solution, 70 per cent alcohol, and 0.5 per cent sodium hydroxide solution, 86.61 per cent of the total protein in the bran was extracted ( $N \times 6.25$ ). The percentages of proteins actually isolated, expressed in terms of the total protein in the bran, were as follows: Albumin, 16.64; globulin, 13.62; alcohol-soluble protein, 31.01 per cent. Analyses showed these proteins to have the following average elementary percentage composition: Albumin, N 15.42, C 53.21, H 6.71, S 1.35; globulin, N 17.76, C 53.43, H 7.40, S 0.91; alcohol-soluble protein, N 15.35, C 54.25, H 6.75, S 1.35.



## THE METABOLISM OF INORGANIC SALTS.

### IV. THE CONTENT OF INORGANIC SALTS IN THE BLOOD IN PREGNANCY, WITH ESPECIAL REFERENCE TO CALCIUM.

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That calcium metabolism is intimately concerned in pregnancy with the development of the fetus is well recognized. Less clearly defined are the alterations in calcium metabolism in the maternal organism and the series of possible metabolic events leading to cessation of the period of uterine life and the expulsion of the fetus. Some years ago an interesting hypothesis relative to these phenomena was promulgated by Bell and Hick. In view of the facts reported by them that the blood calcium increases in the later months of pregnancy and that calcium stimulates the pregnant uterus of animals Bell and Hick (1) suggested that the normal stimulus for the induction of labor is the augmented blood calcium. In confirmation of this work are the observations of Kastle and Healy (2) by whom it was shown that calcium introduced intraperitoneally into pregnant guinea pigs almost invariably led to abortion. However, before such an hypothesis can be regarded as a probability one must first of all be certain that calcium of the blood is at a maximum at the period of labor. This point needs special emphasis since the method of calcium estimation employed by Bell is far from accurate. Of the reported blood calcium figures in pregnancy one fails to find evidence in support of Bell's hypothesis since figures are given which either fall within normal limits or else blood calcium is lower rather than higher during the later months of pregnancy (3).

Most investigators have employed serum rather than whole blood in work on blood calcium on the hypothesis that the corpuscles are calcium-free. It seems to the present authors that a



fallacy may be encountered here since there is *a priori* no reason why under suitable circumstances there should not occur calcium interchange between the corpuscles and the plasma. This view is supported by the observations of Cowie and Calhoun (4) that the corpuscles of the ox and man contain appreciable quantities of calcium. Because of these facts we have preferred to estimate the content of calcium, and indeed of all inorganic salts determined, on whole blood rather than on the serum. The figures given here therefore are not directly comparable to those reported by the more recent investigators

TABLE I  
*Inorganic Salt Content of Blood of Normal Non-Pregnant Women*

Subject	Calcium	Magnesium	Sodium	Potassium	Chlorides	Total phosphates
	mg	mg	mg	mg	mg	mg
44	6.2	3.0	154	297		34
45	7.0	2.5	183	142	284	45
47	6.4		164	177	290	24
48	6.6	2.4	173	188		34
54	6.0	3.7	166	144		38
52	7.6		193	182	304	34
53	8.0		179	210	281	32
60	7.2	2.8	190	228	274	33
63	8.0	2.1	228	149		41
64	5.8		192	200		
Average	6.9	2.7	182	192	286	35

#### *Methods.*

The normal non-pregnant subjects were young unmarried women. The pregnant subjects were normal cases. Blood was drawn from an arm vein into a weighed quantity of water, the blood being determined by difference. In Tables I to IV, therefore, the figures represent milligrams of constituent per 100 gm. of blood. In every case blood was drawn before breakfast, thus eliminating any influence of food intake. The chloride content was estimated by the method of Whitehorn (5). All other constituents were determined by the procedure of Kramer and Tisdall (6).

From the tables it may be seen that the figures for calcium in the blood of normal unmarried non-pregnant women are quite

variable, ranging from 5.8 to 8.0 mg. per 100 gm. of blood. With respect to pregnant women it is quite evident that the average

TABLE II.  
*Inorganic Salt Content of Normal Pregnant Women*

Subject	Period	Calcium	Magnesium	Sodium	Potassium	Chlorides	Total phosphates
		mg.	mg.	mg.	mg.	mg.	mg.
11	3 months.	7 3	3 0	227	210		29
36		6 0	3 1	207	261	284	43
65		7 0	2 1	159	179		40
66		7 8	2 2	182	137	293	35
79		6 9	2 7	213	200		
80		8 2	2 8	205	159	277	
81		8 9	3 1	189	206		
82		6 8	2 7	167	235	283	
83		8 5	2 6		203	287	
88		9 6	1 9	192	169	235	
Average	3 months	7 7	2 6	193	196	276	37
8	4 months	7 3	1 4	200	217	289	45
70		9 7	1 4	166	202		
84		10 1	2 2	197	192	286	
93		7 4	2 9	231	160	283	
Average	4 months	8 6	2 0	198	193	286	
14	5 months	6 5	2 7	197	207	313	31
50			3 2	219	148	297	53
74		7 5	2 2	189	188	281	
78		7 4	2 4	186	197	293	
Average	5 months	7 1	2 6	198	185	296	44
7	6 months	8 0		183	214	297	35
9		5 5	2 9	204	192	300	34
19		7 5	2 1	227	172		39
39		7 6	2 3	237	202	301	31
40		8 1	3 0	197	192	309	44
59		7 1	1 4	174	190		
67		7 4	2 2	227	169	300	24
69		9 7	1 3	160	206	282	
72		10 0	1 8	194	178	298	
58		6 0	3 4	212	262		
Average .	6 months	7 7	2 0	204	198	298	34

TABLE II—Continued.

Subject	Period	Calcium	Magnesium	Sodium.	Potassium	Chlorides.	Total phosphates.
		mg	mg	mg	mg	mg	mg.
25	7 months	7 9		232	243	304	39
46		7 5	2 8	217	217	303	46
51		6 8		201	144	289	44
57		7 4	3 2	122	184	288	38
97		7 2	2 2	213	153		
23		5 7	3 1	219	212	290	30
21		8 9	3 0	210	271	296	28
28		8 6	2 8	239	192	301	37
Average	7 months	7 5	2 8	206	202	296	37
2	8 months	7 0	1 4	213	233	305	23
17		5 8	2 6	238	203		
20		6 9	3 2	222	192		31
22		6 3	2 9	228	163	282	36
32		8 0	1 6	194	231	301	46
71		8 4	1 8	188	201		
85		8 3	2 7	174	225	295	
87		7 3	1 8	189	188	298	
95		8 6	3 9	238	190	298	
15		8 9	3 5	149	236		
Average	8 months	7 6	2 5	203	206	296	34
4	9 months	7 3	1 8	229	173	313	40
10		9 2	2 1	184	228		37
24		6 6	3 5	203	175		27
26		8 4	3 4	317	297	297	34
29		9 4	2 7	239	200	293	
62		8 6	1 1	180	227	292	35
90		7 1	2 5	288	177	301	
Average	9 months.	8 1	2 4	234	211	299	34
77	Term	7 1	2 6	215	208	291	
18		9 9	1 5	233	251	281	41
89		7 9	2 2	174	174		
92		7 6	2 9	185	186	287	
91		11 1	2 4	172	221	301	
96		7 3		192	185	302	
Average	Term.	8 3	2 3	195	204	292	

TABLE II—*Concluded.*

Subject	Period	Calcium	Magnesium	Sodium	Potassium	Chlorides.	Total phosphates.
		<i>mg.</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
12	2 days postpartum.	5 6	3 8	141	281		49
13		8 3		292	199	319	38
30		5 6	3 1	215	236	276	45
41		6 1	3 4	184	215	281	43
42		7 1	2 8	159	174	276	35
55		8.5	2 8	177	280	273	40
73		9 6	2 3	214	218	314	
75		9.0	2 0	158	176	316	
76		7 5	2 5	186	203	288	
Average	2 days postpartum	7 4	2 8	192	220	294	40
16	10 to 12 days postpartum	7 9	3 6	193	229		
37		7 0	2 8	194	221	284	
38		6 9	3 4	190	220	255	39
68		9 7	1 4	196	212	303	
61		7 9	1 4	187	188	284	46
5		6 3	2 6	164	192	267	40
35		6 6	2 9	171	228		45
56		8 2	3 2	229	219	292	44
34		6 1	2 8	172	210	289	44
6		8 2	1 1		311		39
Average .	10 to 12 days postpartum	7 5	2 5	188	223	282	42

blood calcium content is somewhat higher than the corresponding figure in non-pregnant women. From these average figures it may also be observed that calcium has a tendency to become higher at the period of parturition than in the earlier months, although the highest figure is that of 4 months when the average blood calcium is 8.6. After delivery there seems to be a tendency, judging by the average figures, of a return to the lower level.

We are loath, however, to attach much significance to this apparent increase in blood calcium content when the period of gestation approaches term because of the very wide variations encountered when comparison is made of different individuals at the same period of pregnancy. We are inclined to believe that a greater number of estimations would eliminate this apparent

increase of blood calcium. In view of these relatively great individual variations we are justified in concluding that a positive increase in calcium content of the blood with progress of the period

TABLE III  
*Summary (Average Figures)*

Period	Cal- cium	Mag- nesium	Sod- ium	Potas- sium	Chlo- rides	Total phos- phates
	mg	m	mg	mg	mg.	m
Normal	6.9	2.7	182	192	286	35
3 months pregnant	7.7	2.6	193	196	276	37
4 " "	8.6	2.0	198	193	286	
5 " "	7.1	2.6	198	185	296	44
6 " "	7.7	2.0	204	198	298	34
7 " "	7.5	2.8	206	202	296	37
8 " "	7.6	2.5	203	206	296	34
9 " "	8.1	2.4	234	211	299	34
At term	8.3	2.3	195	204	292	
2 days postpartum	7.4	2.8	192	220	294	40
10 to 12 days postpartum	7.5	2.5	188	223	282	42

TABLE IV  
*Ranges in Calcium Content of the Blood.*

Subject	Cases	Calcium
		mg
Normal	10	5.8-8.0
3 months pregnant	10	6.1-9.6
4 " "	4	7.3-10.14
5 " "	4	6.5-7.5
6 " "	11	5.5-10.0
7 " "	8	5.7-8.9
8 " "	10	5.8-8.9
9 " "	7	6.6-9.2
At term	6	7.1-11.1
2 days postpartum		5.6-9.6
10 to 12 days postpartum		6.1-9.7

of pregnancy has not yet been demonstrated. These results therefore fail to lend support to the hypothesis of Bell and Hick that the increased calcium content of the blood at term is of significance in the induction of labor.

The values in pregnancy for the other constituents of the blood determined in general so closely resemble those in the blood of non-pregnant women as to be of little significance.

The variations seen (see Table IV for example) in the individual cases at the same period of pregnancy are sufficiently pronounced to warrant further consideration. In the cases recorded two possible factors bearing upon the problem stand out with considerable prominence. One is the number of previous pregnancies; the other is the age of the individual. Concerning the number of pregnancies, 12 were the first pregnancy, 9 the second, 13 the third, 4 the fourth, 7 the fifth, 3 the sixth, 1 the seventh, 2 the eighth and ninth, 1 the tenth and eleventh, 2 the twelfth, and 1 the sixteenth. Analysis of these data demonstrates, however, that there is no relationship between the number of times a woman has been pregnant and the calcium content of the blood. Similar study with respect to age of the pregnant individual likewise failed to demonstrate any relation of age to calcium content of the blood. The ages under consideration varied from 16 to 40 years; 5 cases were under 20; 12 between 20 and 25, 21 between 25 and 30, 7 between 30 and 35, and 10 between 35 and 40.

#### CONCLUSIONS.

Estimated upon whole blood average figures for calcium content of pregnant women are somewhat higher than those obtained from women not pregnant.

With the course of pregnancy little change can be demonstrated in the calcium of the blood

This fact fails to support the hypothesis of Bell and Hick that increased calcium of the blood at term may be of direct significance in the induction of labor.

The individual variations in the same period of pregnancy cover a relatively wide range, but apparently are not associated with the number of pregnancies or with age.

In general the inorganic constituents of the blood undergo little change during pregnancy.

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## THE METABOLISM OF INORGANIC SALTS.

### V. INORGANIC SALT METABOLISM IN COCAINE POISONING.

BY FRANK P. UNDERHILL AND ERWIN G. GROSS.

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(Received for publication, August 13, 1923.)

Numerous cases of acute poisoning have resulted from the use of cocaine as a local anesthetic. A concentration of 70 to 80 mg. may induce convulsions in man. In dogs, cocaine may produce symptoms of tetany that very closely resemble those following parathyroidectomy. The animals show generalized tremors, increased temperature, and rapid heart rate and respiration. When disturbed they may go into extreme convulsions which usually last from 5 to 10 minutes. Furthermore, as in parathyroid tetany, calcium salts inhibit the symptoms.

Cocaine causes a central stimulation of the motor nerves and also of the sympathetic system. Reduction of calcium in the blood likewise exerts this effect.

In 1921, Mayer (1), working with frogs, found that calcium chloride was antagonistic in action to cocaine. It not only relieved the toxic symptoms, but if administered before the cocaine, prevented the onset of cocaine poisoning. Later, Mayer (2) showed that  $\text{CaCl}_2$  works equally well in the warm blooded animals, both as a preventive and as a cure. Especially marked was its action on the heart. The rapid heart rate following cocaine poisoning was slowed by calcium chloride. Digitalis was found ineffective against the toxic action of cocaine on the heart.

According to the theory of Mayer the antagonistic action of calcium to cocaine is due to a physical change in the colloids of the cells.

Zorn (3), previous to the work of Mayer, demonstrated that potassium salts increase the action of cocaine.



With the symptoms of cocaine poisoning so closely resembling parathyroid tetany, and the action of calcium salts in inhibiting the tetany, we were led to determine whether any changes occur in the inorganic constituents of the blood, similar to those in parathyroidectomy.

#### EXPERIMENTAL.

##### *Procedure.*

Dogs fasted for 48 hours were used as experimental animals. Cocaine HCl was injected subcutaneously in doses sufficient to produce acute toxic symptoms. The blood for analysis was drawn from the external jugular vein. Chlorides were determined by the method of Whitehorn (4), inorganic phosphates by the method of Briggs (5), and calcium, potassium, and sodium by the method of Kramer and Tisdall (6). The magnesium was determined by an adaptation of the Bell-Doisy phosphate method.

The effect upon the inorganic ions following the administration of cocaine HCl is shown in Tables I, II, and III.

No disturbance was noted in the inorganic constituents of the blood, except a slight increase in chloride content. The administration of calcium chloride gave prompt relief from the acute symptoms. In the case of cocaine poisoning, the effect of calcium salts is apparently of a more permanent nature than in parathyroidectomy. In no case have we experienced any resumption of symptoms after their relief by calcium. In parathyroidectomy the beneficial action of calcium salts is only temporary. The action of cocaine is directly on the nervous system, and calcium salts acting as a sedative overcome the stimulating effect of cocaine. The action of calcium is effective against other central nervous stimulants, for example, strychnine (7).

We do not believe it is necessary to explain the action of calcium salts by a physical change in the cell colloids. Increased calcium ions are a central nervous depressant, thus they act antagonistically to the central nervous stimulants, as cocaine and strychnine.

#### SUMMARY.

Cocaine poisoning produces no change in the inorganic constituents of the blood.

TABLE I.

*Inorganic Constituents of the Blood during Cocaine Poisoning.*

Dog 1. Weight 11.0 kilos.

Per 100 gm. whole blood.						Remarks.
Chlorides.	Inorganic phosphates	Calcium	Potassium.	Sodium	Magnesium	
mg.	mg.	mg	mg.	mg	mg.	
301	2 9	5 8 Serum per 100 cc. 11 6	31 2	33 2	3 1	10 00 a m. Normal. 11 45 " Injected subcutaneously 110 mg. cocaine HCl 12 30 p.m Restless, but no other symptoms 3 00 p m Injected subcutaneously 110 mg cocaine HCl 5 00 p m Restless.  10 00 a m. Condition normal 11 00 a m Given 110 mg. cocaine HCl 3 00 p m Given 160 mg cocaine HCl 4 00 p m Restless, but no tremors
312	2 8	5 7 Serum per 100 cc. 11 7	29 1	32 8	2 7	10 00 a m Given 220 mg cocaine HCl 11.30 a m. Given 110 mg. cocaine HCl 12 10 p m Tremors and movement of head, increased temperature Blood sample. The drawing of blood caused the animal to develop violent convulsions, frothing at mouth, these convulsions continued about 5 min. 2.30 p.m Dog still having tremors and head movements.  10 00 a m. Dog apparently normal.

**TABLE II.**  
*Inorganic Constituents of the Blood during Cocaine Poisoning.*  
**Dog 2. Weight 17.0 kilos.**

Per 100 gm. whole blood.						Remarks.
Chlorides.	Inorganic phosphates	Calcium.	Potassium.	Sodium.	Magnesium	
mg.	mg.	mg.	mg.	mg.	mg.	
297	3 05	5 6 Serum per 100 cc. 10 7	28 4	32 4	2 4	9 30 a m. Normal. Blood sample. 10 20 a.m. Injected subcutaneously 340 mg. cocaine HCl. 11 00 a m Dog restless with pupils dilated. 3 00 p.m. Injected subcutaneously 510 mg cocaine HCl. 4 00 p m. Dog very restless, head movements Increased temperature
315	2 80	5 8 Serum per 100 cc 10 7	28 0	31 5	2 7	4 10 p m Blood sample drawn; dog developed extreme convulsions during bleeding, frothing at mouth, rapid respiration, tongue very dark red 4 35 p m Convulsions still continuing Injected subcutaneously 10 cc of 5 per cent CaCl <sub>2</sub> . 4 41 p.m Dog up in cage, respiration normal No convulsions, slightly restless 5 00 p m. Apparently normal.
355	3 1	5 6 Serum per 100 cc 10 7	30 2	31 7	2 4	9 30 a m Dog all right 11 00 a m. Injected subcutaneously 510 mg. cocaine HCl. 11 15 a m Violent convulsions lasting 5 min., but general symptoms continuing 11 30 a.m. Dog died while attempting to draw blood. The blood was drawn immediately from heart.

TABLE III.

*Inorganic Constituents of the Blood during Cocaine Poisoning.*

Dog 3. Weight 14 0 kilos.

Per 100 gm. whole blood						Remarks
Chlorides	Inorganic phosphates	Calcium	Potassium	Sodium	Magnesium	
mg	mg	mg	mg.	mg	mg	
306	2 4	5 8 Serum per 100 cc 10 5	29 4	31 0	2 4	10 15 a.m. Normal. Blood sample 11 40 a m Injected subcutaneously 280 mg cocaine HCl 12 30 p m Dog's head moving continuously up and down, quite rapid opening and closing of mouth
330	Lost	5 9 Serum per 100 cc 10 9	28 1	31 5	2 6	2 45 p m. Blood sample drawn. Tremors and high temperature. 2 50 p m Injected subcutaneously 10 cc of 5 per cent CaCl <sub>2</sub> solution. 3 15 p m Dog practically normal, slightly restless 4 30 p m Still slightly restless.
345	2 6	5 7 Serum per 100 cc 10 4	32 3	31 9	3 1	2 20 p m Injected subcutaneously 14 mg cocaine HCl. 2 45 p m Head movements similar to those of preceding day 3 30 p m Blood sample. 4 15 " Injected subcutaneously 10 cc. of 5 per cent CaCl <sub>2</sub> . 4 40 p.m Not much improvement. 5 00 p m. Nearly quiet. 6 00 " Symptoms apparently disappeared. Next day dog all right.

Calcium salts relieve the toxic symptoms of cocaine, by acting as central nervous depressants.

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## THE INFLUENCE OF HYDRAZINE UPON BLOOD CONCENTRATION AND BLOOD SUGAR CONTENT.\*

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(Received for publication, August 13, 1923 )

In earlier publications (1, 2) it has been shown that the subcutaneous injection of hydrazine sulfate into dogs causes a condition of marked hypoglycemia with an accompanying disappearance of glycogen from the liver and muscles. It is probable that this reduction of carbohydrate in the body is caused by increased combustion (3).

Aside from the influence upon carbohydrate metabolism it was also observed that blood solids (2) are increased under the influence of hydrazine poisoning.

In view of these facts and the further fact that no attempt has been made to follow closely the sequence of events in the blood stream experiments were planned to determine changes in blood sugar content by frequent estimations and to pay particular attention to alterations in blood concentration. The latter condition is regarded as of grave import in a number of pathological states.

As a result of this investigation it may be stated that the injection of hydrazine into dogs causes a marked increase in blood concentration, a condition which may be regarded as a contributing factor for death of the animals.

### EXPERIMENTAL.

#### *Methods.*

Fasting dogs in good physical condition received hydrazine sulfate in a 25 per cent solution by subcutaneous injection in doses

\* The data are taken from the thesis of Dr Samuel Karelitz, Jr., presented in partial fulfillment of the requirements for the degree of Doctor of Medicine, Yale University, 1923

ranging from 50 to 75 mg. per kilo. Blood sugar was determined by the method of Folin and Wu, and hemoglobin according to the procedure of Smith and Cohen, the blood being drawn from a vein.

*Influence of Fasting upon Blood Sugar and Hemoglobin Contents.*

Inasmuch as dogs poisoned with hydrazine refuse food control experiments were carried through upon normal fasting dogs; a typical protocol, Table I, being illustrative of the results obtained. From this table it is quite apparent that the changes observed in

TABLE I  
*Influence of Fasting upon Blood Sugar and Hemoglobin Contents.*

Day of fasting	Time	Hemoglobin in percentage of normal	Glucose per 100 cc blood
			<i>mg.</i>
1	a m.	100	93
	p m	102	88
2	a m	105	86
	p m	112	90
3	a m.	109	92
	p m	101	87
4	a m.	103	90
	p m.	98	98
5	a m	105	95
	p m	101	100
6	a m	106	89

both blood sugar and hemoglobin contents are too small to be of any practical significance. It is, therefore, evident that fasting alone is without significant influence upon the problem under discussion.

*Influence of Hydrazine upon Blood Sugar and Hemoglobin Contents.*

In Tables II, III, and IV are given results, typical of many others, which show the influence of hydrazine upon blood concen-

TABLE II.

*Influence of Hydrazine upon Blood Concentration and Blood Sugar Content.  
Subcutaneous Injection of 50 Mg. per Kilo of Hydrazine Sulfate into a Dog of  
5.5 Kilos.*

Hours after hydrazine injection.	Hemoglobin in percentage of normal	Glucose per 100 cc blood.
		<i>mg.</i>
Before.	100	107
1	103	143
2	103	152
6	105	237
8	108	263
12	122	156
22	128	125
24	130	97
27	140	58
Died.		

TABLE III

*Influence of Hydrazine upon Blood Concentration and Blood Sugar Content.  
Subcutaneous Injection of 75 Mg per Kilo of Hydrazine Sulfate into a Dog of  
10 Kilos*

Hours after hydrazine injection	Hemoglobin in percentage of normal	Glucose per 100 cc blood
		<i>mg.</i>
Before	100	98
5	118	108
22	135	115
29	145	120
46	188	81
54	156	71
Died.		

*Subcutaneous Injection of 75 Mg per Kilo of Hydrazine Sulfate into a Dog of  
3 5 Kilos.*

		<i>mg.</i>
Before.	100	115
2	95	122
4	107	113
22	135	172
25	127	168
48	144	129
53	109	131
71	119	114
Recovered.		



tration and blood sugar content. The increased concentration of the blood is very striking, the degree of hypoglycemia is less evident, and although significant, is not as marked as the results of earlier work. It is also of interest to note that when the animals recovered, which occasionally occurs, increased blood concentration, although high, is transitory, whereas when a fatal outcome ensues blood concentration usually becomes progressively greater up to the time of death. From our experience with other pathological states in which blood concentration is a prominent feature we are inclined to the belief that in hydrazine poisoning a concentrated blood must be regarded as an important contributing factor in the fatal outcome.

TABLE IV

*Influence of Hydrazine upon Blood Concentration and Blood Sugar Content.  
Subcutaneous Injection of 75 Mg per Kilo of Hydrazine Sulfate into a Dog of  
10.5 Kilos*

Hours after hydrazine injection	Hemoglobin in percentage of normal	Glucose per 100 cc blood
		mg
Before.	100	114
4	102	135
20	141	116
31	148	112
45	162	93
52	178	76
Died		

Another factor which is of considerable significance is the early state of hyperglycemia without any evidence of glycosuria when tested by the ordinary reduction tests. Generally the degree of hyperglycemia here attained causes the appearance of sugar in the urine and why glucose fails to evidence itself in hydrazine poisoning is not quite clear since there is no indication under these circumstances of any decrease in renal integrity.

Blood concentration in hydrazine poisoning may be due to one of two causes. In the first place, since the drug exerts an almost specific (4) influence upon the liver it may evoke the mechanism leading to blood concentration described by Lamson (5) as peculiar to the liver, or, in the second place, the concentrated blood may be ascribed to less specific factors, as loss of fluid and failure

of fluid intake. Until more certain knowledge of the exact mechanism is available we are inclined to regard the second set of factors as sufficient explanation for the appearance of the phenomenon under discussion. When a dog is poisoned with hydrazine more or less constant salivation, vomiting, and diarrhea are in evidence, and there is an inability for at least 48 hours to retain either food or water. We believe that these conditions are sufficient to account for the increase in blood concentration.

From the data it is quite evident that there is no direct relationship between the degree of blood concentration and the blood sugar content, although usually the highest blood concentration occurs when hypoglycemia is lowest. The degree of hypoglycemia in these animals was much less marked than in those reported in previous papers, the cause being unknown.

#### CONCLUSIONS.

Hydrazine poisoning in dogs causes a marked increase in blood concentration which is ascribed to fluid loss and failure of fluid retention. It is probable that concentration of the blood may be a prominent factor contributing to the usual fatal outcome of this intoxication.

There is no necessary relationship between the degree of blood concentration and the extent of hypoglycemia induced by hydrazine, although generally blood concentration is at its maximum when hypoglycemia is most marked.

Previous to the notable decrease in blood sugar content a transitory condition of hyperglycemia prevails without the appearance of sugar in the urine and without any evidence of a kidney lesion.

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## STUDIES IN CARBOHYDRATE METABOLISM.

### XXI. THE RELATION OF SUGAR EXCRETION TO RENAL INTEGRITY.\*

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(Received for publication, August 13, 1923.)

The relation of the kidney membrane to blood sugar content has always presented interesting problems. Thus, for example, it is believed that phlorhizin exerts a specific action on the kidney producing glycosuria even though blood sugar content remains within normal limits. On the other hand, glycosuria usually occurs as a result of a hyperglycemia. The renal threshold for sugar is a variable factor, for during pregnancy it is lowered at times, and in the progress of chronic diabetes there is manifested on the part of the renal filter a tendency to hold back sugar even though a simultaneous hyperglycemia may be present. Neubauer considers that there exists a relationship between increased blood pressure and hyperglycemia (1). In sixteen cases of nephritis with increased tension Hopkins (1) noted a moderate increase in blood sugar in eleven. In ten other cases without tension, three were hyperglycemic. In all cases of nephritis he observed a gradual return of the blood sugar to normal subsequent to oral administration of glucose.

The clinical suggestion that in diabetes of long standing the renal membrane becomes less permeable to sugar has led us to test the converse problem; namely, the influence of a definite kidney lesion to blood sugar content.

\* The data are taken from the thesis of Dr. Gustav Wilens, presented in partial fulfillment of the requirements for the degree of Doctor of Medicine, Yale University, 1923.

TABLE I.

*Influence of Sodium Tartrate Nephritis upon the Blood Sugar of a Fasting Rabbit.*

Rabbit G. Weight 3,700 gm Water given.

Day	Hour	Water intake	Urine.		Blood		Remarks
			Volume	Sugar	Sugar	Hemo-globin in percentage of normal.	
		cc.	cc	mg.	mg.		
6	9 a m.				178	100	
	12 n.					105	
	5 p m.	200	160	271	192	119	
7	9 a m.				144	99	
	12 n					100	
	5 p m.	256	172	137	151	77	

2.8 gm of tartaric acid neutralized with NaOH in 10 cc of H<sub>2</sub>O injected subcutaneously.

8	9 a m.				163	84	Paralysis of hind legs
	12 n.					82	
	5 p m	308	10	6	166	96	
9	9 a m.				190	84	Same. Tremors. Ataxia
	12 n					83	
	5 p m	250	15	2	172	84	
10	9 a m.				250	78	Same.
	12 n.					72	
	5 p m	195	148	242	208	69	
11	9 a m				203	64	Same Glycosuria.
	12 n.					60	
	5 p m	145	129	268	303	54	
12	9 a m	0	39	111			Found dead. Glycosuria

*Autopsy* —Swollen, pale, soft kidneys Pneumonia of right lung with scattered patches in left lung 39 cc of urine in bladder. Large organized clot in sacrum over sacral nerve plexus, and attached to left femoral vessels.

## EXPERIMENTAL.

*Methods.*

Tartrate nephritis was induced in normal fasting rabbits by subcutaneous administration of sodium tartrate in doses of 50 to 75 mg. per kilo. Estimations were made on blood from a marginal ear vein of blood sugar, by the method of Folin and Wu, and of hemoglobin by the Cohen and Smith procedure. Sugar in the urine was determined by the Benedict and Osterberg method.

*Influence of Tartrate Nephritis upon Blood Sugar Content of the Fasting Rabbit.*

In this series of experiments (see Tables I, II, and III) the animals received tartrate at the end of the 2nd day of fasting. As soon as 24 hours subsequent to the injection of tartrate a distinct rise in blood sugar content occurs, although this is only transitory. Later there is a distinct fall to normal or below and simultaneously there is a decided increase in the urine sugar output. Just before or during the period of high blood sugar, one observes a decrease in urine volume and sugar excretion. This observation coincides with the statement by Underhill, Wells, and Goldschmidt (2) that sodium tartrate inhibits glycosuria in phlorhizinized animals. Underhill (3) showed that this inhibition occurs even without the use of phlorhizin. Upon the basis of these facts the quantities of sugar eliminated by the normal kidney must be retained by the damaged kidney, being manifested by the appearance of hyperglycemia. The results detailed in the accompanying tables tend to confirm this. However, one must bear in mind the possibility that extrarenal factors play a part in the production of hyperglycemia represented above. Underhill, Wells, and Goldschmidt (2) claimed that "Neither the liver nor the adrenal exhibits any detrimental effect from the injection of tartrates." However, one may have altered function without any obvious anatomical correlation (4). Moreover, if one assumes that tartrate has a specific extrarenal influence in the production of hyperglycemia would one not expect the high point of excess blood sugar to occur simultaneously with the greatest concentration of the drug? This would probably occur about 24 hours after injection.

TABLE II.

*Influence of Sodium Tartrate Nephritis upon the Blood Sugar of a Fasting Rabbit.*

Rabbit H. Weight 2,100 gm. Water given.

Day	Hour	Water intake	Urine		Blood		Remarks
			Volume	Sugar	Sugar	Hemo-globin in percentage of normal	
		cc	cc	mg	mg		
6	9 a m				133	100	
	12 n					177	
	5 p m	84	30	58	192	106	
7	9 a m				144	86	
	12 n					90	
	5 p m	68	27	47	135	85	

2.1 gm of tartaric acid neutralized with NaOH in 10 cc of H<sub>2</sub>O injected subcutaneously

8	9 a m				128	85	
	12 n.					86	
	5 p m	224	19	15	144	88	
9	9 a m				166	87	Ataxia Tremors
	12 n					85	
	5 p m	210	0	0	158	77	
10	9 a m				133	78	Severe diarrhea.
	12 n					79	
	5 p m	86	0	0	132	70	
11	9 a m				123	64	Same.
	12 n					55	
	5 p m	200	0	0	131	37	
12	9 a m.				148	20	Dyspnea. Glycosuria.
	12 n.					25	
	5 p m	0	8	16	307	12	

Died following convulsions and coma.

*Autopsy*—Soft, swollen, pale, granular kidneys. About 25 cc. of straw-colored fluid in peritoneal cavity. Intestine contained fluid and walls appeared gelatinous. Bladder empty.

An inspection of the tables reveals no such phenomenon. In fact it is later than the first 24 hours that the peak of glycemia is reached. Also, the anuria, the diuresis with fall in blood sugar

TABLE III.

*Influence of Sodium Tartrate Nephritis upon the Blood Sugar of a Fasting Rabbit.*

Rabbit B. Weight 3,000 gm. Water given

Day	Hour	Water intake	Urine		Blood		Remarks
			Volume	Sugar	Sugar	Hemo-globin in percentage of normal	
		cc	cc	mg.	mg		
3	9 a m				193	100	
	12 n					99	
	5 p m	50	65	46	198	101	
4	9 a m				196	100	
	12 n.					102	
	5 p m.	60	50	50	208	104	

2.25 gm. of tartaric acid neutralized with NaOH in 10 cc of H<sub>2</sub>O injected subcutaneously

5	9 a m				145	95	
	12 n					92	
	5 p m.	80	15		156	89	
6	9 a.m				151	78	
	12 n.					70	
	5 p m	45	0	0	142	65	Anuria
7	9 a m				189	78	
	12 n.					74	Diarrhea.
	5 p m.	100	0	0	192	66	Anuria.

Died in coma following day.

*Autopsy* —Soft, pale, edematous kidneys No urine in bladder About 20 cc. of straw-colored fluid in peritoneal cavity.

level, and the secondary rise tend to favor the hypothesis that the hyperglycemia is due to altered kidney function.

On the other hand, it is well known that uremia, dyspnea, edema with chloride retention, and other conditions may be asso-



ciated with hyperglycemia. It is possible that tartrate nephritis does away with the ability of the kidneys to remove excess of acids formed in a state of increased metabolism as in the animals under discussion. Acidosis and dyspnea would result. At times these rabbits were quite obviously dyspneic. According to Peabody "the acidosis of renal disease runs parallel to the accumulation of waste nitrogen in the blood and to the ability of the individual to eliminate phenolsulphonaphthalein." Underhill and Blatherwick (5) and Potter and Bell (6) have shown that a retention of nitrogen and a decrease in excretion of phenolsulfonephthalein can occur in tartrate nephritis. From these facts it is possible that both renal and extrarenal factors play a rôle in the establishment of a condition of hyperglycemia in rabbits made nephritic by sodium tartrate injection.

In Rabbit H, Table II, it may be seen that the rise in blood sugar subsequent to tartrate injection is not as great as that occurring with the other animals. If one may assume that the intestines take over in part the function of the damaged kidneys, the explanation for this diverse result is apparent. In this animal diarrhea was most severe and at autopsy the intestines contained fluid, the walls being remarkably transparent and gelatinous in appearance. Kleiner (7) does not believe that the gastrointestinal tract compensates very significantly in the absence of kidney function, a view which is in direct contrast to that of MacCallum who states (8): "that in the absence of the kidney the intestines assume a supplementary excretory function."

The concentration of hemoglobin tends to fall in all the animals under observation. If this dilution of the blood noted above takes place without significant anemia, then the blood sugar values given in the tables are actually higher than they appear. But as a notable uniform dilution has been observed in control experiments, one must conclude that anemia as well as hydremia takes place in tartrate nephritis. Both anemia and hydremia may be present in certain types of human nephritis.

Tartrate nephritis, especially in the acute stages, is essentially a tubular affair. However, it is possible that the vascular portion may be influenced by the intracapsular pressure produced by the reaction to the injury. Glomerular lesions are rarely seen during the acute stage. Indefinite accumulations of serum in Bowman's

capsules have been observed during the period of chronicity (9). The anuria produced is the result of obstruction of the uriniferous tubules by extensive plugs composed of epithelial and leucocytic debris, and the increased intracapsular pressure. From the tables it may be seen that at certain times the kidneys are quite capable of removing sugar from the blood, although the greater portion of the tubules has been damaged. It must, therefore, be concluded that the glomeruli normally are active in the elimination of glucose, or that under the circumstance of epithelial necrosis they have taken over the function of the cells lining the tubules. If the glomeruli normally remove sugar from the blood stream, either very small amounts come through, or larger quantities are reduced by reabsorption by the cells, because the sugar in normal urine is very small compared to the blood sugar. If this be true, the facts fit nicely with the "reabsorption theory" of Cushny, and would explain, in a measure, why chronic diabetics with elevated blood sugar levels do not present glycosuria. It is known that diabetics with nephritis present albuminuria with glycosuria. When the albuminuria clears up, in some cases the glycosuria ceases, and it may be that a similar mechanism is concerned with this observation (10).

*Influence of Subcutaneous Injections of Glucose upon Blood Sugar Content in Tartrate Nephritis.*

If in tartrate nephritis an elevation of blood sugar occurs, it would be of interest to determine the influence upon blood sugar content of glucose introduced into the nephritic animal. In Tables IV and V such data are detailed. In the two examples cited one animal received a subcutaneous injection of glucose 1 day subsequent to the injection of tartrate, the other on the 3rd day. Following the injection of the tartrate one sees again an elevation of blood sugar. In Rabbit D, Table IV, the rise in blood sugar is noticed after introduction of the sugar, but this is no more marked than that observed in other experiments where tartrate only had been administered. This rise is coincident with a drop in urine volume and urinary sugar. Then there appears an increase in kidney function, namely diuresis, and perhaps glycosuria with a fall in blood sugar level. Again, as the kidney shuts

TABLE IV

*Influence of a Subcutaneous Injection of Glucose upon the Blood Sugar of a Fasting Rabbit, Previously Made Nephritic by a Subcutaneous Injection of Sodium Tartrate.*

Rabbit D. Weight 3,200 gm Water given

Day	Hour.	Water intake	Urine		Blood		Remarks
			Volume	Sugar	Sugar	Hemo-globin in percentage of normal	
		cc	cc	mg	mg		
16	9 a m.				136	100	
	12 n					101	
	5 p m	106	110	85	133	93	
17	9 a m				98	103	
	12 n					105	
	5 p m.	134	54	56	105	102	

24 gm. of tartaric acid neutralized with NaOH in 10 cc of H<sub>2</sub>O injected subcutaneously

18	9 a m				119	97	
	12 n.					94	
	5 p m	186	46	58	144	84	

16 gm of glucose in 160 cc of H<sub>2</sub>O injected subcutaneously.

19	9 a m				213	85	
	12 n				270	84	
	5 p m	36	0	0	175	86	Drowsy
20	9 a m				135	92	
	12 n					87	
	5 p m	44	273	470	150	92	Diarrhea.
21	9 a m				219	117	
	12 n.					119	
	5 p m.	104	142	473	312	120	Conjunctivitis.
22	9 a m				235	113	
	12 n				370	112	Ataxia.
	5 p m	74	0	0	377	111	Very drowsy.
23	9 a m.	0	52	208			Found dead.

*Autopsy.*—All organs appeared normal except kidneys, which were pale, soft, and swollen 52 cc of urine found in bladder

TABLE V.

*Influence of a Subcutaneous Injection of Glucose upon the Blood Sugar of a Fasting Rabbit, Previously Made Nephritic by a Subcutaneous Injection of Sodium Tartrate.*

Rabbit C. Weight 2,600 gm. Water given.

Day.	Hour	Water intake.	Urine.		Blood.		Remarks
			Volume	Sugar	Sugar.	Hemo-globin in percentage of normal	
		cc	cc	mg.	mg		
8	9 a.m.				149	100	
	12 n.					101	
	5 p m	60	85	85	142	102	
9	9 a.m.				158	92	
	12 n.					80	
	5 p.m	116	80	71	142	74	

1.95 gm. of tartaric acid neutralized with NaOH in 10 cc. of H<sub>2</sub>O injected subcutaneously.

10	9 a m				108	75	
	12 n					67	
	5 p m.	158	70	56	142	65	
11	9 a.m.				151	68	
	12 n.					66	
	5 p.m	58	52	32	218	74	
12	9 a.m.				200	67	
	12 n.					66	
	5 p.m.	110	72	54	270	66	

13 gm. of glucose in 130 cc. of H<sub>2</sub>O injected subcutaneously.

13	9 a.m.				333	57	
	12 n.					64	
	5 p.m.	68	114	129	270	61	
14	9 a.m.				291	74	
	12 n.					76	
	5 p.m.	106	50	58	284	65	
15	9 a.m.				180	66	
	12 n.					67	
	5 p.m.	100	50	98	213	65	
20					180		Albumin in urine.

down the blood sugar becomes elevated to an even higher level. During the increase in urine volume one observes a slight concentration of the blood and an increase of water intake.

Rabbit C reacted less intensely to the injection of the tartrate, anuria never being present, although kidney function was decidedly diminished and blood sugar was raised. A supplementary rise in blood sugar followed the glucose injection with a subsequent increase of kidney function and a fall in blood sugar. 10 days after the injection of tartrate blood sugar was still elevated. From these experiments it is quite obvious that following tartrate nephritis there appears a state of hyperglycemia in rabbits and at times there may be increased kidney function as far as the elimination of sugar is concerned. In other words, the threshold to sugar appears to be alternately elevated and lowered in tartrate nephritis. An injection of glucose results in a still higher elevation of blood sugar which is in excess of that noted with tartrate alone.

#### CONCLUSIONS.

In experimental tartrate nephritis the renal threshold to glucose is altered, irregularly favoring the blood or the urine.

The administration of glucose to nephritic animals is followed by still higher levels of blood sugar content.

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## GLUCOKININ.

### AN APPARENT SYNTHESIS IN THE NORMAL ANIMAL OF A HYPOGLYCEMIA-PRODUCING PRINCIPLE. ANIMAL PASSAGE OF THE PRINCIPLE.

#### THIRD PAPER.

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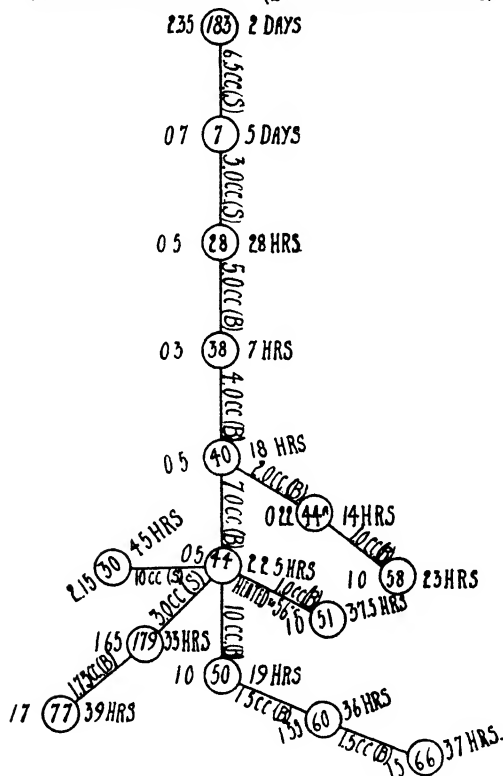
#### INTRODUCTION.

When it was found that hypoglycemia of a marked degree developed at such irregular intervals in a series of normal rabbits injected with various plant extracts, it was decided to observe the effect of the blood of a reacting animal upon other normal animals. It was at once found that the whole blood (defibrinated) or the serum separated from defibrinated blood taken from a reacting animal either before or after death produced, with remarkable uniformity, hypoglycemia in normal rabbits. The reaction in the second animal was manifested as a rule within 24 hours even though the reaction in the first animal might have been as long delayed as several weeks. It was then found that the whole blood (defibrinated), or serum obtained from serum- or defibrinated blood-inoculated rabbits would also produce hypoglycemia in a third animal. Having established, therefore, that animal passage was possible, a series of experiments was at once planned and carried out in order to test out just how far animal passage could be carried and to determine if an actual increase in potency occurred as the number of animal passages was increased. The results of the preliminary experiments on this new problem are described and discussed below. The phenomenon of animal passage is illustrated graphically in Charts 1 to 5.



As the main purpose of the experiments, the results of which are outlined below, was to determine the extent to which animal passage of this principle was possible, blood samples for reinjection were not taken until the animal under observation had

ONION GLUCOKININ (200 GRAMS ONION TOPS)



## CHART 2

manifested very definite signs of hypoglycemia—very low blood sugar, extreme weakness, or convulsions. A certain number of experimental animals died before blood samples were taken. If in such instances the animal was still quite warm when found, a blood analysis was made, but if the animal was cold no blood analysis was made but a blood sample was taken direct from the heart and reinjected into another normal animal. The blood was in practically every instance defibrinated by whipping. The whole



blood after defibrination was then used for injection into other animals, but in a few experiments serum separated from the defibrinated blood was used. The injections were made under

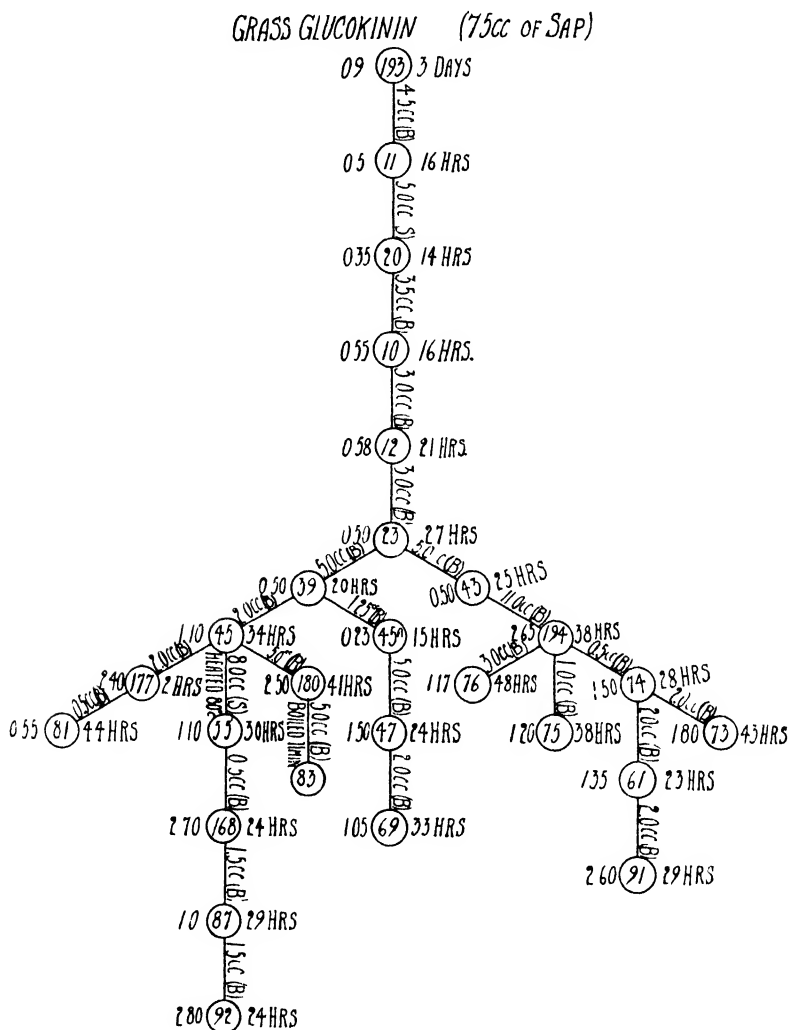


CHART 3.

the skin on the dorsal surface. Those instances in which intravenous injections were made are indicated in the tables.

Blood samples for sugar estimation were withdrawn from a marginal ear vein at various times from the injected animals. Heart puncture was resorted to in a few special cases.

Sugar determinations were made by the Shaffer-Hartmann method (2).

### 12CC SERUM FROM TREATED DEPANCREATIZED DOG

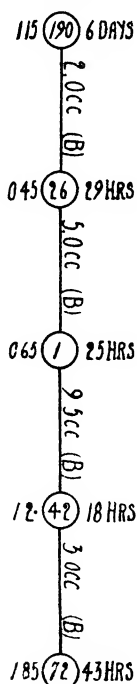


CHART 4.

The rabbits used were stock animals which had been fed hay and oats. No preliminary fasting period was imposed, but the animals were not fed during the period of observation unless otherwise indicated.

### DISCUSSION AND FURTHER RESULTS.

The following conclusions may be drawn from results shown in Table I. (1) The blood of a rabbit, dying with hypoglycemia as a result (either direct or indirect) of previous inoculations

with a plant extract, is capable of producing profound hypoglycemia and death when such blood is injected into other normal rabbits. (2) When normal rabbits are so inoculated and later die with hypoglycemia the blood of these animals has similar properties. (3) Animal passage would seem to be possible to

*PURIFIED GLUCOKININ - 150cc GRASS SAP*

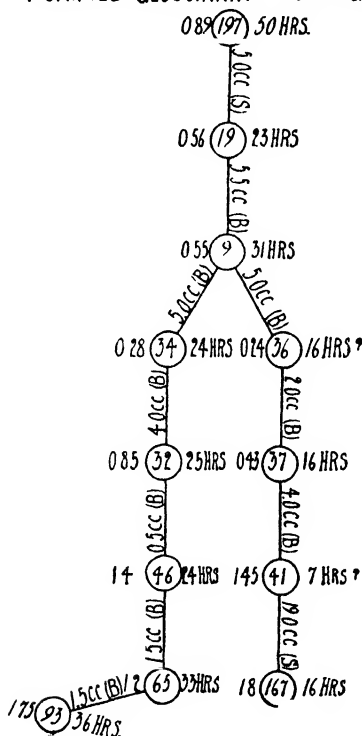


CHART 5

an indefinite extent. (4) An organism as the causative agent is very improbable. (5) The characteristic symptoms—low blood sugar, extreme weakness and general atonia, and convulsions—may be antidoted temporarily by the administration of glucose, but the ultimate fatal issue has not been offset as yet by such a procedure.

While the results of the above experiments are very clear-cut the explanation of this remarkable phenomenon is not apparent.

It is a matter of great interest to know if the blood of insulinated rabbits has similar properties to the passage blood used in these experiments. When the typical symptoms of insulin hypoglycemia in normal rabbits were first noted by the writer, it was found that the blood of a convulsed animal did not produce hypoglycemia when administered to another normal rabbit. The second animal was observed for only a short period at that time. This experiment has therefore been repeated. The result obtained is given in Table II.

It is also true, as the experiment outlined in Table III will indicate, that normal rabbits which have been fasted for a period of 5 days or longer may manifest somewhat similar symptoms (extreme weakness, collapse, and convulsions) and die in a condition of hypoglycemia. The results obtained in the above experiment cannot, therefore, be attributed entirely to the fact that the first animal in the series had received insulin.

It is possible that the time of duration of a condition of low blood sugar may be in some manner associated with the appearance in the blood stream of the reacting animal of an hypoglycemia-producing principle. To test out this possibility rabbits were given relatively enormous doses of insulin and when symptoms developed they were antidoted by glucose administration. It was found in these experiments that such an insulinated animal would continue to develop typical symptoms of hypoglycemia at intervals of a few hours. For a time the symptoms could be absolutely controlled by glucose administration, but the animal finally died. It would appear, therefore, that the behavior of an animal receiving an extreme overdose of insulin is quite like the behavior of the blood passage animals described in Tables I, II, and III. The symptoms can be antidoted only temporarily by glucose administration. See Table IV.

#### *Spontaneous Hypoglycemia.*

It is also worthy of note that on occasions a stock rabbit may develop symptoms of low blood sugar and die in convulsions. The liver of such an animal has been shown on examination to be full of cysts, and on microscopic examination *Coccidia oviforme* have been found and extensive degenerative changes have been

TABLE I.  
*Animal Passage.*

Date	Animal	Sex	Weight	Time.	Blood sugar	Remarks
Series 1.						
Apr 16	Rabbit 123.	♀	2,210 gm	6 30 p m.	0 100	5 cc. purified yeast glucokinin.
" 16				12 00 m.	0 087	
" 17				3 00 a m.	0 090	
" 17				6 00 "	0 096	
" 17				9 00 "	0 096	
" 17				2 00 p m.	0 093	
" 17				12 00 m	0 103	
" 18				6 00 a m.	0 103	
" 18				11 00 "	0 103	Returned to run to feed.
May 4				10 00 "	0 087	Found lying in a listless state. Bled from heart. Liver almost glycogen-free.
" 20			1,230	3 00 p m	0 024	7.5 cc. defibrinated blood from No. 123. Found dead.
" 20	Rabbit 2.		700	3 30 "		
" 25				8 00 a m.		
" 20	Rabbit 17.		515	3 30 p m		8 cc. defibrinated blood from No. 123.
" 21				3 00 "	0 030	Convulsions. Bled from heart.
" 21	Rabbit 16.		600	4 00 "		7 cc. defibrinated blood from No. 17.
" 22				8 00 "	0 030	At death.

May 23	Rabbit 27.		490	9 00 a.m.	3.5 cc defibrinated blood from No. 16.
" 24				8 00 "	Found dead.
" 24	Rabbit 24.		950	8 30 "	2.5 cc. defibrinated blood from No. 27.
" 25				8 30 "	At death
" 25	Rabbit 182.	♀	1,900	11 45 p m.	5 cc. defibrinated blood from No. 24.
" 26				6 00 a m.	
" 26				10 45 "	0 097
" 26				8 30 p m.	0 080
" 27				8 00 a m.	0 065
					At death.
" 25	Rabbit 56.		996	12 00 m	2.5 cc defibrinated blood from No. 2.
" 26				6 30 a m.	0 097
" 26				12 40 p m.	0 077
" 26				11 00 "	0 020
					At death.
" 27	Rabbit 78.	♀	1,300	10 00 "	1.5 cc. defibrinated blood from No. 182.
" 28				7 00 a m.	0 106
" 28				12 00 n.	0 087
" 28				7 00 p m.	0 080
" 29				2 00 a.m.	0 073
" 29				1 30 p m.	0 070
					At death.
" 27	Rabbit 70.	♀	1,500	10 00 "	11.5 cc. defibrinated blood from No. 56.
" 28				6 00 a m.	0 114
" 28				1 20 p m.	0 068
" 29				12 00 n.	0 042
					At death.

TABLE 1—Continued.

Date	Animal.	Sex	Weight gm	Time	Blood sugar	Remarks
Series 1—Continued						
May 30	Rabbit 101.	♀	2,050	12 00 m.	per cent 0 108	1.5 cc defibrinated blood from No. 78.
" 31	"			2 00 a m.	0 130	
" 31	"			7 00 "	0 114	
" 31	"			10 30 "	0 077	
June 1				4 50 p.m.	0 020	At death.
" 1				7 30 "		
" 2	Rabbit 137.	♂	1,325	11 30 a m.		0.05 cc defibrinated blood from No. 101. Original blood was diluted with 19 vols distilled water.
" 3	"			10 45 "	0 042	Found in typical convulsions. Bled out at once.
" 2	Rabbit 148.	♀	1,400	11 30 "		0.1 cc. defibrinated blood from No. 101.
" 4	"			1 30 "	0 024	Typical convulsions. Bled out at once.
" 2	Rabbit 145.	♀	1,400	11 30 "		0.25 cc. defibrinated blood from No. 101.
" 3	"			7 00 p m	0 018	Typical convulsions. Bled out at once.
" 2	Rabbit 141.	♀	1,350	11 30 a m.		0.5 cc. defibrinated blood from No. 101.
" 3	"			10 30 "		Found dead.
" 2	Rabbit 134.	♂	1,500	11 30 "		1 cc. defibrinated blood from No. 101.
" 3	"			10 30 "		Found dead.

June 3	Rabbit 133.	♀	1,200	12 00 n.	0 024	0 05 cc. defibrinated blood from No. 137. Typical convulsions. Bled out at once.
" 4				9 00 a. m.		
July 16	Rabbit 45.	♂	2,043	12 00 n.		1.25 cc of defibrinated blood from No. 101. This blood diluted with 19 vols. distilled water. Stood in ice chest since June 1.
" 17				5 40 p.m.	0 076	Animal normal.
" 18				7 30 a. m.	0 068	" very weak.
" 18				11 00 "	0 050	Died in a typical convulsion. Bled out at once.
" 18				11 30 "		
" 18	Rabbit 48.	♀	2,346	4 00 p. m.		4 cc. defibrinated blood from No. 45.
" 19				6 30 a. m.	0 093	
" 20				1 10 "	0 042	Animal very weak, lying with limbs stretched out.
" 20				1 40 "		10 cc. 25 per cent glucose subcutaneous.
" 20				1 50 "		Animal able to sit up.
" 20				4 30 "		Convulsions. 10 cc. 25 per cent glucose subcutaneous.
" 20						Animal prostrate for 45 min. Then sat up, but still very weak. Could not hop around without falling over.
" 20				5 00 "		5 cc. 25 per cent glucose.
" 20				7 45 "		Died without convulsions.
" 18	Rabbit 47.	♀	2,241	4 00 p. m.		3 cc. defibrinated blood from No. 45 subcutaneous.
" 19				6 40 a. m.	0 090	Animal normal.
" 19				7 15 p. m.	0 024	Became very weak and died without typical convulsions.
" 18	Rabbit 49	♀	2,118	4 00 "	0 100	2 cc. defibrinated blood from No. 45 subcutaneous.
" 19				6 45 a. m.	0 100	



Date	Animal	Sex	Weight	Time	Blood sugar	Remarks
Series I—Concluded						
July 19	Rabbit 49.			10 25 p m	per cent 0 010	Animal prostrate 10 cc 25 per cent glucose subcutaneous.
" 19				10 35 "	0 010	2 min later, severe convulsion and death. Sugar evidently not absorbed.
" 20	Rabbit 58	♀	1,857	11 30 a m.		4 cc defibrinated blood from No 45. Injected as a suspension. Blood had previously been autoclaved 20 min. Pressure of 6 lbs. maintained 10 min.
" 21				3 00 "	0 083	Animal very weak
" 21				6 30 p m	0 024	" collapsed 10 cc. 25 per cent glucose intravenous.
" 21				7 30 "		Animal sitting up at 10 min. Able to use limbs, but still very weak
" 21				8 20 "		Violent convulsions. No blood obtainable. Gave 10 cc. 25 per cent glucose intravenous. Died as injection was being made.
" 20	Rabbit 61.	♀	2,159	11 30 a m.		3 cc defibrinated blood from No. 45. Injected as suspension. Blood previously autoclaved as in above experiment
" 21				6 00 "	0 077	
" 22				9 00 "	0 042	Animal drowsy and very weak. 10 cc. 25 per cent glucose injected.
" 22				9 20 "		Animal sitting up
" 22				9 50 "		Hopped around.
" 22				10 00 "		Convulsions. 2 cc glucose intravenous. Convulsions continued and animal died in 20 min.

July 20	Rabbit 62.	♀	2, 212	11 30 a m		2 5 cc defibrinated blood from No. 45. Injected as suspension. Blood previously autoclaved as in above experiment.
" 21				12 00 n.	0 093	
" 22				7 30 p m.	0 077	
" 22				10 00 "		
" 23				12 30 a m.		Very weak 10 cc 25 per cent glucose subcutaneous. Considerable improvement in 10 min.
" 23				3 30 "	0 108	Very weak again 10 cc. 25 per cent glucose subcutaneous. No improvement. 17 cc more glucose given in course of next 3 hrs. No improvement.
" 24	Rabbit 69.	♀	1, 659	4 00 p m.		At death.
" 25				6 00 a m.	0 093	
" 25				6 30 p m.	0 060	
" 26				4 45 a m.		2 cc defibrinated blood from No. 45. Animal allowed to feed throughout experiment.
" 26				7 00 "		Convulsions 10 cc. 25 per cent glucose subcutaneous. Partial recovery.
" 26				8 15 "		Very weak. 5 cc 25 per cent glucose. Condition did not change.
						Died suddenly

TABLE II.  
*Insulin.*

Date.	Animal	Sex	Weight	Time	Blood sugar	Remarks.
Series I.						
July 17	Rabbit 39.	♂	1,911	11 00 a.m.	per cent	3 cc old insulin = approximately 30 units. Violent convulsions. Continued on and off till dead. Died.
" 17				1 10 p.m.		
" 17				3 40 "		
" 17	Rabbit 40.	♀	2,300	4 00 "		12 cc defibrinated blood from No. 39.
" 18				8 00 a.m.	0 080	
" 19				7 00 "	0 090	
" 20				7 00 "	0 087	. . .
" 21				6 30 "	0 083	
" 23				6 40 "	0 068	
" 24				6 40 "	0 074	Aborted during night.
" 25				6 15 "	0 036	Very weak.
" 28				7 30 "	0 032	Died suddenly. Slight spasm just previous to death.
" 17	Rabbit 41.		1,000	4 00 p.m.		4 cc. defibrinated blood from No. 39.
" 23				7 00 a.m.	0 058	
" 23				4 30 p.m.	0 018	
" 17	Rabbit 35.	♀	1,241	4 00 "		Became very weak and finally developed convulsions. Died at once.
" 24				6 30 a.m.	0 100	
						6 cc. defibrinated blood from No. 39.

July 25				0 083	Very weak all this day.
" 26				0 058	Slight spasm and death
" 26				0 010	
" 25	Rabbit 85.	♀	1,444		2 cc defibrinated blood from No. 40
" 26				0 093	Listless 10 cc 25 per cent glucose subcutaneous. No recovery.
" 27					At death.
" 27				0 042	
" 23	Rabbit 71.	♂	1,469		3 cc. defibrinated blood from No. 41.
" 24				0 090	Convulsions 10 cc 25 per cent glucose subcutaneous. No recovery.
" 27					Blood from heart.
" 27				0 052	
" 25	Rabbit 90.	♀	1,173		2 cc defibrinated blood from No. 71.
" 26				0 100	Appeared weak. 10 cc. 25 per cent glucose subcutaneous. Recovered.
" 27				0 058	Very weak Head rolling from side to side. 3 cc. 25 per cent glucose intravenous. Immediate improvement.
" 27				0 050	Showing symptoms again. 5 cc. 25 per cent glucose subcutaneous. Sat up in 10 min.
" 27					Convulsions. 5 cc. 25 per cent glucose intravenous. Recovered at once.
" 27				0 053	Listless again. 5 cc. 25 per cent glucose subcutaneous. Partial recovery only, then gradually weakened again.
" 27					

TABLE II—Continued.

Date	Animal	Sex	Weight	Time	Blood sugar	Remarks
Series 1—Concluded						
			gm		per cent	
July 27	Rabbit 90			10 45 p m		5 cc 25 per cent glucose subcutaneous. Improved again.
" 28				12 30 a m		Could sit up in 15 min
" 28				3 00 "		5 cc 25 per cent glucose subcutaneous
" 28				3 30 "		5 " 25 " "
					0 026	Slight convulsions and death
Series 2						
May 14	Rabbit 183.	♂	2.337	6 00 p m		25 cc crude extract of green onion tops = 200 gm. fresh material
" 15				9 00 a m	0 125	
" 15				5 00 p m	0 096	
" 16				9 00 a m.	0 124	
" 16				6 00 p m	0 118	
" 17				10 00 a m	0 148	
" 17				12 00 n		Found dead Bled out.
" 17	Rabbit 7.		720	3 30 p m		6 5 cc blood serum from No 183.
" 19				11 00 a m	0 103	
" 20				11 00 p m	0 070	
" 21						Very weak all day.
" 22				1 00 a m	0 036	Convulsions. Bled from heart.
" 22	Rabbit 28.		512	8 30 "		3 cc blood serum from No. 7.
" 23				12 30 p m	0 020	Animal in convulsions. Bled from heart.

# J. B. Collip

May 23	Rabbit 38.	305	12 45 p m		5 cc defibrinated blood from No. 28.
" 23			8 00 "	0 040	Very weak Bled from heart. Died.
" 23	Rabbit 40.	500	8 00 "		4 cc defibrinated blood from No 38.
" 24			2 00 "	0 045	Convulsions Bled from heart
" 24	Rabbit 44.	500	2 30 "		7 cc defibrinated bood from No. 40.
" 25			1 00 "	0 032	At death.
" 24	Rabbit 44A.	220	2 30 "		2 cc. defibrinated blood from No. 40.
" 25			8 00 a m		Found dead
" 25	Rabbit 30	2,150	10 00 p m		10 cc blood serum from No 44.
" 26			5 00 a m.	0 124	
" 26			9 00 "	0 077	
" 26			7 45 p m.	0 067	
" 27			7 00 a m	0 073	
" 27			10 30 "	0 073	
" 28			2 15 "	0 065	At death.
" 25	Rabbit 179.	1,650	10 00 p m.		3 cc blood serum from No 44.
" 26			6 00 a m.	0 103	
" 26			8 00 p m.	0 103	
" 26			12 00 m.	0 073	
" 27			6 30 a m.	0 060	At death.
" 25	Rabbit 58.	970	11 45 p m.		1 cc. defibrinated blood from No. 44A.
" 26			6 50 a m.	0 097	

TABLE II—Continued.

Date.	Animal.	Sex	Weight	Time	Blood sugar	Remarks
Series 2—Concluded						
May 26	Rabbit 58.		gm	1.10 p m	percent 0 083	
" 26				9 00 "	0 045	
" 26				11 00 "	0 015	At death.
" 25	Rabbit 51.	♀	1,009	11 00 "		10 cc. defibrinated blood from No 44. This blood was kept at 56-63°C for 20 min before injection.
" 26				5 00 a m	0 108	
" 26				1 30 p m	0 080	
" 26				7 45 "	0 067	
" 27				12 35 a m	0 026	Convulsions and death.
" 25	Rabbit 50.	♂	1,050	11 00 p m		10 cc defibrinated blood from No 44 Subcutaneous.
" 26				7 00 a m.	0 099	
" 26				10 00 "	0 070	
" 26				6 00 p m.	0 045	Semicomatose. Bled from heart. Died.
" 27	Rabbit 60.	♀	1,550	10 00 "		1 5 cc defibrinated blood from No. 50. Subcutaneous.
" 28				2 00 a m.	0 103	
" 28				6 00 "	0 118	
" 28				9 30 "	0 083	
" 28				4 30 p m	0 073	
" 29				10 10 a m.	0 015	Died.

				Series 3.			
May 27	Rabbit 77.	♀	1,625	10 00 p m		1 75 cc defibrinated blood from No. 179.	
" 28				5 30 a.m.	0 100		
" 28				1 25 p.m.	0 087		
" 29				1 40 a m.	0 045	At death.	
" 30	Rabbit 66.	♀	1,550	12 00 n.		1 5 cc. defibrinated blood from No. 60.	
" 31				9 00 a.m.	0 073		
" 31				1 30 p m.	0 015	Convulsions and death.	
May 15	Rabbit 193.	♀	892	5 00 p m	0 090	15 cc crude grass extract = 75 cc. sap.	
" 16				10 00 "	0 077		
" 17				6 00 "			
" 18				10 00 a m.	0 063		
" 18				7 00 p m	0 032	At death.	
" 18	Rabbit 11.		500	8 30 "		4 5 cc defibrinated blood from No. 193.	
" 19				12 00 m	0 062	Very weak. Bled from heart. Died shortly after.	
" 19	Rabbit 20.		355	1 00 p m.		5 cc. blood serum from No 11.	
" 20				1 00 a.m.	0 065	Animal very weak.	
" 20				8 00 "		Found dead.	
" 20	Rabbit 10		550	11 00 "		3 5 cc. defibrinated blood from No. 20.	
" 20				12 00 n	0 068	Animal very weak.	
" 21				8 00 a.m.		Found dead.	
" 21	Rabbit 12.		580	8 30 "		3 cc. defibrinated blood from No. 10.	
" 22				4 00 "		Very weak.	
" 22				6 00 "		Found dead.	



TABLE II—Continued

Date	Animal	Sex	Weight	Time	Blood sugar	Remarks.
Series 3—Continued.						
May 22	Rabbit 23		500	8 30 a m	percent	3 cc defibrinated blood from No 12.
" 23				4 00 p m.		Animal very weak. Bled out.
" 23	Rabbit 39.		500	4 00 "		5 cc. whole blood from No. 23.
" 24				12 00 n.		Convulsions and death.
" 23	Rabbit 43.		500	4 00 p m.		5 cc whole blood from No. 23
" 24				5 00 "	0 032	At death
" 24	Rabbit 45	♀	1,100	2 30 "		2 cc defibrinated blood from No. 39
" 25				6 00 "	0 077	
" 25				10 15 "	0 032	Typical convulsions and death.
" 24	Rabbit 45A		220	2 30 "		1 25 cc. defibrinated blood from No 39.
" 25				1 00 "	0 045	Very weak. Bled out
" 25	Rabbit 177.	♀	2,400	11 30 "		2 cc defibrinated blood from No. 45.
" 26				6 00 a m.	0 106	
" 26				9 00 "	0 080	
" 26				6 30 p m.	0 077	
" 26				8 00 "	0 058	Convulsions Bled out.
" 25	Rabbit 47.	♀	1,475	10 30 "		5 cc. defibrinated blood from No. 45A.
" 26				5 30 a m.	0 108	

May 26				10 00 a.m.	0 087	
" 26				7 00 p.m.	0 070	
" 26				11 00 "	0 020	At death.
" 24	Rabbit 194.	♀	2,645	5 30 "		11 cc defibrinated blood from No. 43.
" 25				4 30 a.m.	0 070	
" 26				8 30 "	0 040	At death.
" 26	Rabbit 74.	♀	1,465	1 30 p.m.		0 5 cc defibrinated blood from No. 194.
" 26				7 30 "	0 124	
" 27				2 30 a.m.	0 077	
" 27				7 30 "	0 065	
" 27				6 00 p.m.	0 015	Comatose. Bled out.
" 26	Rabbit 75.	♂	1,188	1 30 "		1 cc defibrinated blood from No. 194.
" 26				7 00 "	0 099	
" 27				2 30 a.m.	0 096	
" 27				7 30 "	0 087	
" 27				9 30 "	0 083	
" 28				3 30 "	0 032	At death
" 26	Rabbit 76.	♂	1,170	1 30 p.m.		3 cc defibrinated blood from No. 194.
" 26				7 30 "	0 103	
" 27				10 30 a.m.	0 073	
" 28				2 05 p.m.	0 036	At death
" 25	Rabbit 180.	♀	2,500	10 30 "		5 cc defibrinated blood from No. 45.
" 26				7 00 a.m.	0 097	

TABLE II—Continued

Date	Animal	Sex	Weight	Time	Blood sugar	Remarks
Series 3—Continued.						
May 26	Rabbit 180		gm	10 30 a.m.	per cent 0 065	At death.  8 cc blood serum from No 45. This serum was kept at 80°C for 12 min The clot was beaten up in 10 cc. of saline solution and the whole injected.
" 26				5 00 p.m.	0 083	
" 27				7 30 a.m.	0 080	
" 27				9 15 "	0 067	
" 27				3 00 p.m.	0 018	
" 25	Rabbit 55.	♀	1,070	11 30 "		At death.  8 cc blood serum from No 45. This serum was kept at 80°C for 12 min The clot was beaten up in 10 cc. of saline solution and the whole injected.
" 26				6 00 a.m.	0 103	
" 26				11 30 "	0 083	
" 26				7 30 p.m.	0 080	
" 27				2 00 a.m.	0 080	
" 27				5 30 "	0 026	At death.
" 26	Dog.	♀	8,400	12 00 n.	0 150	Depancreatized at 4 00 p.m. May 25. 8 cc. blood serum from Rabbit 45.
" 26				12 00 m.	0 158	Animal vomited continuously after operation.
" 27				4 30 a.m.	0 112	Died suddenly. Blood sample taken at once.
" 27				10 00 p.m.		7 cc defibrinated blood from above dog.
" 28	Rabbit 71.		750	1 00 a.m.	0 103	
" 28				6 00 "	0 097	

May 28				12 20 p m.	0 068	
" 28				6 30 "	0 073	
" 29				7 00 "	0 024	At death.
" 27	Rabbit 81.	550		11 00 "		0 5 cc defibrinated blood from No. 177.
" 28				2 00 a m.	0 108	
" 28				6 00 "	0 114	
" 28				10 00 "	0 073	
" 28				5 00 p m	0 062	
" 28				7 00 "		Died. Blood sample lost.
" 27	Rabbit 168.	♂	1,600	10 00 "		0 5 cc defibrinated blood from No. 55.
" 28				3 30 a m.	0 118	
" 28				6 30 "	0 121	
" 28				11 15 "	0 087	
" 28				6 30 p m	0 055	Convulsions and death.
" 28				10 00 "	0 025	
" 28	Rabbit 87.	♂	1,050			1 5 cc defibrinated blood from No 168.
" 29				1 30 a m	0 093	
" 29				7 00 "	0 068	
" 29				3 30 p m.	0 048	Convulsions and death.
" 30	Rabbit 92.	♀	2,790	12 00 m.		1 5 cc. defibrinated blood from No. 87.
" 31				7 00 a.m.	0 070	
" 31				12 30 p m.	0 020	Convulsions and death.
" 27	Rabbit 83.		700	10 00 "		5 cc. blood serum from No. 180. Added to 5 cc. saline solution and heated in boiling water bath for 11 min. before injection.
" 28				10 00 a.m.	0 099	

TABLE II—Continued.

Date	Animal	Sex	Weight	Time	Blood sugar	Remarks
Series 3—Concluded						
May 28	Rabbit 83.		gm	5 00 p.m.	per cent 0 080	
" 29				10 00 a.m.	0 018	At death.
" 27	Rabbit 69.	♂	1,050	10 00 p.m.		2 cc defibrinated blood from No. 47.
" 28				7 00 a.m.	0 103	
" 29				1 20 "	0 055	
" 29				7 00 "	0 030	At death
" 27	Rabbit 61.	♀	1,350	10 00 p.m.		2 cc defibrinated blood from No. 74.
" 28				9 00 "	0 032	Typical convulsions and death.
" 28	Rabbit 91.	♂	2,600	9 30 "		2 cc defibrinated blood from No. 61.
" 29				3 00 "	0 073	
" 30				1 20 a.m.	0 048	Convulsions and death.
" 27	Rabbit 73	♂	1,800	9 00 p.m.		2 cc defibrinated blood from No. 74.
" 28				1 00 a.m.	0 103	
" 28				5 30 "	0 097	
" 28				10 00 "	0 087	
" 29				4 15 "	0 032	At death.

May 15	Rabbit 190.	♀	1,156	12 00 n		12 cc blood serum of depancreatized dog taken post mortem. Animal treated with onion extract. Lived 66 days Last injection May 2.
" 15				5 30 p m.	0 121	
" 16				10 00 a m.	0 097	
" 19				9 00 "	0 118	Put back in run to feed
" 21				8 00 "		Found dead.
" 21	Rabbit 26.		450	10 00 "		2 cc defibrinated blood from No. 190
" 23				3 30 p m		Animal very weak. Bled from heart.
" 23	Rabbit 1.		655	4 00 "		5 cc defibrinated blood from No. 26.
" 24				3 00 "	0 080	
" 24				5 00 "	0 045	Animal very weak. Bled from heart.
" 24	Rabbit 42.	♀	1,200	5 30 "		9 5 cc defibrinated blood from No. 1.
" 25				5 00 "	0 080	
" 26				5 00 a m	0 062	
" 26				11 00 "	0 026	At death.
" 26	Rabbit 72.	♂	1,850	12 00 n		3 cc. defibrinated blood from No. 42.
" 26				5 30 p m.	0 115	
" 27				3.30 a m.	0 083	
" 27				7.30 "	0 080	
" 27				10 30 "	0 080	
" 28				6.40 "	0.020	At death.

Series 4—Continued.

Date	Animal.	Sex	Weight	Time	Blood sugar	Remarks
May 26	Dog	♀	14 1 kilos	12 00 n.	per cent 0 220	8 cc defibrinated blood from Rabbit 42
" 26				12 00 m	0 165	Animal depancreatized at 3 p m. May 25.
" 27				10 00 a m.	0 180	
" 27	Rabbit 84		800	11 00 p m		6 cc. defibrinated blood withdrawn from above dog at 10 a m. May 27.
" 28				11 30 a m	0 108	
" 28				6 30 p m.	0 068	
" 29				10 40 a m.	0 042	At death.

Series 5

May 18	Rabbit 197.	♀	890	2 45 p m	2 5 cc purified extract of lawn grass	} = 150 cc. sap.
" 18				3 45 "	2 5 " "	
" 18				4 50 "	2 5 " "	
" 18				5 00 "	0 118	(The active principle was removed from fresh sap by ammonium sulfate and the precipitate purified by alcohol.)
" 19				9 00 a m	0 085	Animal collapsed 2 gm. glucose in 10 cc. water subcutaneous. Partial recovery in course of next half hr.
" 20				5 00 p m.	0 058	Animal found dead.
" 20				9 00 "		
" 20	Rabbit 19.		560	9 30 "		5 cc. blood serum from No. 197.
" 21				8 30 a m.	0 052	Very weak. Bled out from heart.

May 21	Rabbit 9.		545	8 30 a m.	0 045	5 5 cc. whole blood from No. 19. Very weak. Bled from heart.
" 22				3 00 p m.		
" 22	Rabbit 34		279	3 30 "		5 cc defibrinated blood from No. 9.
" 23				4 00 "		Very weak. Bled from heart.
" 23	Rabbit 32.		850	4 00 "		4 cc defibrinated blood from No 34.
" 24				5 30 "	0 042	Convulsions. Bled out.
" 24	Rabbit 46.	♀	1,400	6 00 "		15 cc. defibrinated blood from No. 32.
" 25				9 00 a m.	0 058	
" 25				6 30 p.m.	0 024	At death.
" 22	Rabbit 36.		239	3 30 "		5 cc. defibrinated blood from No. 9.
" 23				7.30 a m.		Died.
" 23	Rabbit 37.		430	9 00 "		2 cc. defibrinated blood from No. 36.
" 24				1 00 "	0 036	Typical convulsions. Bled out.
" 24	Rabbit 41.	♂	1,450	1 00 "		4 cc. whole blood from No. 36 intravenous. 2 cc. subcu- taneous
" 24				8 30 "	0 225	Died.
" 24	Rabbit 167.	♀	1,812	5 00 p m.		19 cc. blood serum from No. 41.
" 25				9.00 a m.	0 032	Died.
" 27	Rabbit 65.	♀	1,150	10.00 p m.		1.5 cc. defibrinated blood from No. 46.
" 28				1 30 a.m.	0 103	



TABLE II—Concluded.

Date.	Animal	Sex.	Weight	Time	Blood sugar	Remarks.
Series 5—Concluded						
May 28	Rabbit 65		gm.	Percent		
" 28				5 30 a.m.	0 103	
" 29				1 30 p.m.	0 063	
				7 00 a.m.	0 036	At death.
" 30	Rabbit 93	♀	1,750	12 00 n		15 cc defibrinated blood from No 65.
" 31				1 15 a.m.	0 083	
" 31				4 00 p.m.	0 080	
June 1				12 25 a.m.	0 024	At death

noted. The blood of such an animal has produced hypoglycemia when injected into another normal rabbit. This is illustrated by the experiments shown in Table V.

### *Guanidine Hypoglycemia.*

Guanidine hypoglycemia has been previously observed by Watanabe (3). A normal rabbit injected with guanidine sulfate may develop in the course of many hours symptoms of hypoglycemia simulating in a general manner the effect of certain plant extracts. Two series of animal passages of a hypoglycemia-producing principle have been made, using the blood of a guanidinized animal. The results of these experiments are shown in Table VI.

### *Nature of the Active Principle.*

Experiments already quoted (Series 1) would indicate that a thermostabile substance in the blood of a reacting animal is responsible for the production of the specific effect in the inoculated animal (in a series of animal passages). Experiments shown in Table VII would indicate that the substance is not only thermostabile but in all probability a fairly simple organic compound.

Since spontaneous hypoglycemia may occur at times and also since prolonged fasting may lead to death with hypoglycemia some of the earlier experiments quoted are open to criticism. It might be necessary, therefore, to exclude all experiments (bearing an animal passage) in which definite symptoms were not manifested within 2 or 3 days and in which coccidial infection was not excluded. The liver and other tissues of all experimental animals are now being submitted to microscopic examination. In only a few instances has the liver of an experimental animal been found absolutely free from small white spots on the surface. These surface markings have varied from two or three to thirty or forty in number. The lobular tissue, however, in the majority of cases shows no marked abnormality, while in other instances degenerative changes of varying degrees have been noted. Professor Revell has kindly undertaken a detailed histological study of certain tissues of experimental animals dying with hypoglycemia induced in various ways. His results will be reported at a later date.

TABLE III  
*Fasting Hypoglycemia.*

Date.	Animal.	Sex.	Weight gm	Time	Blood sugar per cent	Remarks
July 30	Rabbit 80.		2,178			Fasted 5 days. Found dead.
" 30	Rabbit 114.	♀	2,178	12 00 n. 5 30 a m	0 052	3 cc defibrinated blood from No. 80. Convulsions and death.
Aug. 2						
" 1	Rabbit 87.	♂		3 00 p m.	0 026	Found dead; still warm. Fasted 7 days.
" 2	Rabbit 175.	♀	1,936	12 30 " 7 20 "		3 cc defibrinated blood from No. 87. Convulsions. Sugar injected subcutaneous. Convulsions ceased Animal in state of collapse.
" 4				8 30 "	0 080	Died.
" 1	Rabbit 92.	♂		10 20 "	0 038	Found collapsed. Had fasted 7½ days 5 cc. 25 per cent glucose intravenous. Partial recovery. Could sit up, but very weak.
" 2				2 00 a.m.	0 032	Died.
" 2	Rabbit 133.	♀	958	12 30 p m. 7 30 "		1 cc. defibrinated blood from No. 92. Convulsions. 10 cc. 25 per cent glucose subcutaneous Recovered in 10 min.
" 3						Convulsions. 5 cc. 25 per cent glucose subcutaneous. Recovered in 10 min.
" 3				11.45 "		

Aug. 4			2 50 a m.			Convulsions 10 cc. 25 per cent glucose subcutaneous. Partial recovery.
" 4			7 45 "		0 110	Very weak. 5 cc. glucose. No recovery.
" 4			9 00 "			Died.
" 2	Rabbit 86.	♀	11 00 p m.			Very weak. Had fasted 7½ days. 5 cc. 25 per cent glucose subcutaneous. Recovered.
" 3			6 45 a.m.		0 032	Died.
" 2	Rabbit 158.	♂	12 30 p m	1,635		2 cc. defibrinated blood from No. 86.
" 4			10 00 "		0 073	Very weak
" 5			7 00 a.m.		0 060	Convulsions. 5 cc. 25 per cent glucose. Partial recovery.
" 5			8 00 "		0 010	Died.
" 5	Rabbit 84.		2 00 "		0 052	Convulsions and death. Fasted 8½ days.
" 3	Rabbit 142.	♂	11 30 "	1,784		3 cc. defibrinated blood from No. 84.
" 5			12 45 "		0 008	Weak.
" 5			5 20 p m		0 026	Collapsed. 10 cc. 25 per cent glucose. No recovery. Died 10 min. later.

TABLE IV.  
*Insulin.*

Date	Animal.	Sex	Weight	Time	Blood sugar	Remarks
Series 2.						
July 18	Rabbit 94.		gm 1,000	11 00 a m.	per cent	1 cc highly purified insulin = approximately 20 units.
" 18				1 10 p m.		Convulsions. Gave 4 cc 25 per cent glucose subcutaneous.
" 18				4 50 "	0 042	Almost immediate recovery.
" 19				12.40 a m.	0 045	On verge of convulsions. 10 cc 25 per cent glucose.
" 19				1 20 "		Very weak. 5 cc. 25 per cent glucose.
" 19				8 10 "		Fully recovered
" 19				8 15 "		Convulsions 5 cc. 25 per cent glucose.
" 19				4 00 p m.		Recovered.
" 20				2 40 a m.	0 032	Mild convulsive seizure. Allowed to feed. Recovered without sugar injection.
" 20	Rabbit 59.	♂	2,860	11 30 "		Convulsions and death.
" 21				6 00 "	0 090	10 cc defibrinated blood from No. 94.
" 21				11 00 "	0 058	Showing symptoms of hypoglycemia. Legs spread out from body.
" 21				11 50 "		Very weak 10 cc. 25 per cent glucose.
" 21				12 00 n.		Considerable improvement, but animal still weak and unsteady when made to move about.
" 21				12 40 p m.		5 cc. 25 per cent glucose intravenous. Sat upright immediately after.

July 21			2 15 p m			Violent convulsions. 15 cc 25 per cent glucose subcutaneous. No recovery. Died.
" 21			2 30 "			
" 21	Rabbit 67.	♂	7 30 "	1,313		1 cc. defibrinated blood from No 59.
" 22			7 00 a m.		0 124	
" 23			4 30 "		0 087	
" 23			2 00 p m			Found collapsed. Attempted to get blood from ear. Violent convulsions at once 5 cc. 25 per cent glucose intravenous.
" 23			2 15 "			Animal sitting up.
" 23			3 40 "			" again collapsed. Died as intravenous glucose was being administered.
" 23	Rabbit 70.	♀	4 15 "	1,514		1 cc. defibrinated blood from No. 67.
" 24			5 00 a m.		0 074	
" 25			7 00 p.m.			Convulsions. 10 cc. 25 per cent glucose subcutaneous.
" 25			7 20 "			Sitting up, but very weak.
" 25			9 45 "			10 cc. 25 per cent glucose subcutaneous as animal much weaker.
" 25			10 05 "		0 052	Died.
" 26	Rabbit 101.	♀	6 00 "	1,680		2 cc. defibrinated blood from No. 70.
" 27			9.15 a m.		0.010	Violent convulsions. Died at once.

Blood  
sugar

## Series 3

		Rabbit 63.	♀	gm.		per cent	
July 21				3,000	12.30 p.m.	60 units insulin subcutaneous.	
" 21					2 30 "	Convulsions. 15 cc. 25 per cent glucose. Complete recovery in 10 min.	Complete re-
" 21					4 45 "	Convulsions. 15 cc 25 per cent glucose. Recovered in 10 min.	Recovered in
" 21					7 10 "	Convulsions. 15 cc. 25 per cent glucose. Recovered in 10 min.	Recovered in
" 21					11 20 "	Very weak. 15 cc 25 per cent glucose. Recovered in 10 min	Recovered in
" 22					5.30 a.m.	Violent convulsions. 15 cc 25 per cent glucose. Died at once.	Died
" 25		Rabbit 77.	♀	1,651	4 00 p m.	2 cc. defibrinated blood from No. 63.	
" 26					6 45 a.m.		
" 27					4.00 "	Violent convulsions. 10 cc. 25 per cent glucose subcutaneous.	
" 27						No recovery. Died at once.	

## Series 4.

July 2	♂	12 45 p m	2 cc. insulin approximately 40 units.	
" 2		3 25 "	Convulsions. 5 cc. 25 per cent glucose. Recovered.	us

July 21					6 30 p.m.			Convulsions. 15 cc. 25 per cent glucose subcutaneous. Recovered.
" 21					10 40 "			Very weak 15 cc. 25 per cent glucose subcutaneous. Recovered.
" 22					2 30 a.m.			Very weak. 15 cc 25 per cent glucose subcutaneous. Recovered
" 22					6 25 "			Very weak 15 cc 25 per cent glucose subcutaneous Recovered
" 22					7 30 p m.	0 073		Very weak 15 cc. 25 per cent glucose subcutaneous. Only partial recovery.
" 23					3 45 a.m.			Very weak 15 cc 25 per cent glucose subcutaneous.
" 23					6 45 "			Able to sit up in 15 min.
" 23					8 50 "			Violent convulsions 9 cc 25 per cent glucose intravenous. No recovery, listless till death.
" 23					9 40 "			Died.
" 23					2 30 p m.			3 cc. defibrinated blood from No. 64.
" 24				♀ 1,067	4 20 a.m.	0 026		Very weak. 5 cc 25 per cent glucose subcutaneous. Only slight improvement.
" 24					5 00 "			5 cc. 25 per cent glucose.
" 24					6.15 "			5 " 25 " "
" 24					6 45 "	0 100		Died No convulsions
" 25					4 00 p m.			2 cc. defibrinated blood from No. 65.
" 26				♀ 2,779	11 45 "	0 032		Collapsed. 10 cc. 25 per cent glucose subcutaneous. Only slight improvement.
" 27					3 15 a.m.			10 cc. 25 per cent glucose. No change.
" 27					4.20 "	0 056		Died.



TABLE V.  
*Spontaneous Hypoglycemia.*

Date.	Animal	Sex	Weight gm	Time	Blood sugar percent	Remarks
June 12	Rabbit.			1 00 p m	0 036	A new stock rabbit found collapsed. Bled out.
"	Rabbit 169.		719	4 00 "		3 5 cc blood of above rabbit subcutaneous.
" 16				12 50 a m.	0 038	Convulsions Bled out.
July 27	Rabbit.			11 30 "	0 018	Small stock rabbit in state of collapse. Bled out. Liver full of cysts <i>Coccidia oviforme</i> found on microscopic examination.
" 25	Rabbit 89.	♀	1,521	5 00 p m.		3 cc. serum from blood of above rabbit.
" 26				5 30 a m	0 087	
" 27				4 20 "	0 077	
" 27				7 25 p m	0 010	Convulsions. Sugar administered, but animal died at once.

TABLE VI.  
*Guanidine.*

Date.	Animal	Sex	Weight	Time	Blood sugar	Remarks.
Series 1.						
July 10	Rabbit 26.	♂	2,347 gm	11 30 a m		50 mg guanidine sulfate injected subcutaneous.
" 10				1 00 p m.	0 134	
" 10				3 00 "	0 116	
" 10				5 00 "	0 116	
" 12				3 00 "	0 077	
" 13				4 00 "	0 108	
" 14				8 00 a m.		Found dead.
" 14	Rabbit 41.	♂	2,246	12 30 p m		5 cc defibrinated blood from No 26.
" 17				4 30 a m.		Violent convulsions. 10 cc. 25 per cent glucose subcutaneous.
" 17				5 10 "		Recovered, but still weak.
" 17				8 15 "		Convulsions. 15 cc. 25 per cent glucose subcutaneous.
" 17				8 30 "	0 018	No improvement. Convulsions continued. Animal died 8 30 a m.
" 17						Blood from heart.
" 17	Rabbit 46.	♀	2,284	11 00 "		10 cc. defibrinated blood from No. 41.
" 17				6 30 p m.	0 106	
" 18				7 15 a m.	0 065	
" 19				7 30 "		Convulsions. 10 cc. 25 per cent glucose subcutaneous.
" 19				7 55 "	0 055	No improvement.
" 19						Blood from heart at death.

TABLE VI—Continued.

Date.	Animal	Sex	Weight	Time	Blood sugar	Remarks
Series 1—Concluded						
July 19	Rabbit 55.	♀	3,225 gm	12 00 n.	per cent	6 cc. defibrinated blood from No. 46.
" 20				6 20 a.m.	0 110	
" 21				5 30 "	0 070	
" 21				1 30 p.m.	0 032	Animal very weak.
" 21				1 50 "		10 cc 25 per cent glucose subcutaneous.
" 21				2 00 "		Convulsions No recovery.
July 19	Rabbit 54	♀	2,980 gm	12 00 n		3 cc defibrinated blood from No 46.
" 20				6 00 a.m	0 100	
" 20				1 00 p.m.		Suddenly developed violent convulsions. 10 cc. 25 per cent glucose subcutaneous. No recovery. Bled out 1.15 p.m.
" 20				1 15 "	0 018	
" 20	Rabbit 57.	♀	2,057 gm	6 30 "		2 5 cc defibrinated blood from No. 54.
" 21				5 00 a.m.	0 080	
" 22				5 30 "	0 018	Very weak 10 cc 25 per cent glucose subcutaneous.
" 22				6 10 "		Attempted to sit up, but unable to do so.
" 22				6 25 "		Died.
" 20	Rabbit 60.	♀	2,058 gm	6 30 p.m.		1 5 cc. defibrinated blood from No. 54.
" 21				6 00 a.m.	0 080	
" 22				1 45 p.m.	0 036	Animal had been drowsy for some hours previous.
" 22				1 50 "		Violent convulsions. 10 cc. 25 per cent glucose subcutaneous Died immediately after injection.

## Series 2.

July	17	Rabbit 37.	♂	1,573	11 00 a m.	0 018	100 mg guanidine sulfate subcutaneous. Animal very weak. 10 cc. 25 per cent glucose subcutaneous. Much improved Able to run around. Animal became extremely weak 10 cc. 25 per cent glucose subcutaneous Very slow in rallying. Sitting up Hair standing up in a peculiar manner. Animal very weak. Violent convulsions 10 cc 25 per cent glucose subcutaneous. No recovery. Bled out.
"	18				12 50 "		
"	18				12 55 "		
"	18				1 10 "		
"	18				8 40 "		
"	18				10 00 "		
"	18				1 45 p m	0 032	
"	18				2 10 "		
"	18	Rabbit 50	♀	1,252	4 00 "	0 097	3 cc defibrinated blood from No 37.
"	19				6 00 a m	0 073	
"	20				6 30 "		Lying stretched out with head rolling from side to side. 5 cc 25 per cent glucose subcutaneous.
"	20				2 30 p m		Running around.
"	20				2 45 "		Collapsed 5 cc 25 per cent glucose subcutaneous. Convulsed for 5 min. immediately after, then slowly recovered.
"	20				6 30 "		
"	20				8 20 "		Convulsions 5 cc. 25 per cent glucose subcutaneous.
"	20				8 40 "		Considerably recovered.

TABLE VI—*Concluded.*

Date	Animal	Sex	Weight	Time	Blood sugar	Remarks
Series 2—Concluded						
July 20	Rabbit 50			9 45 p m	<i>per cent</i>	Very weak again. 5 cc. 25 per cent glucose subcutaneous.
" 20				10 10 "		Sitting up again
" 20				11 05 "		Lying listless 5 cc 25 per cent glucose subcutaneous. No improvement.
" 21				12 45 a m.	0 055	At death.
" 18	Rabbit 53.	♀	2,600	4 00 p m		5 cc defibrinated blood from No. 37.
" 20				6 30 a m.	0 100	
" 21				5 00 "	0 090	
" 21				6 20 p m.		Animal listless No blood obtainable. 10 cc. 25 per cent glucose subcutaneous. Only slight improvement.
" 21				7 45 "		Collapsed. 10 cc. 25 per cent glucose subcutaneous. No improvement.
" 21				8 00 "		Died.
" 23	Rabbit 66.	♀	2,809	3 00 "		2 cc. defibrinated blood from No. 53.
" 24				10 00 "	0 036	Convulsions. 10 cc. 25 per cent glucose subcutaneous. Died immediately after injection.

TABLE VII

Date	Animal.	Sex.	Weight	Time	Blood sugar	Remarks
Dialysis of principle						
July 30	Rabbit 124.	♀	1,171	4 30 p m.	per cent	20 cc distilled water out of total of 35 cc. in which a fish bladder containing 15 cc passage blood had been immersed for 2 hrs.
Aug. 2				3 35 "	0 042	Collapsed. 3 cc 25 per cent glucose intravenous.
" 2				7 05 "		Animal very weak 5 cc. 25 per cent glucose subcutaneous.
" 3				7 30 a m		Kept alive 12 hrs by glucose. 25 cc. in all injected.
July 31						Convulsions were noted once at 4 10 a.m.
Aug. 1	Rabbit 139.	♀	1,142	11 00 "		Died.
" 1				12 00 n		15 cc = remainder of dialysate.
" 2				3 40 p m.	0 042	Convulsions 5 cc. 25 per cent glucose intravenous.
				2 15 a m.		Recovered.
						Convulsions. 5 cc 25 per cent glucose intravenous.
						Recovered.
						Convulsions and death
Tungstic acid filtrate.						
July 27	Rabbit 105.	♀	1,485	2 15 p m.		30 cc filtrate after precipitation of protein from passage blood by tungstic acid. Folin-Wu technique.
" 28				6 00 a.m.	0 090	
" 29				3 30 "	0 087	
" 30				5 00 "	0.068	

TABLE VII—Continued.

Date.	Animal	Sex	Weight	Time	Blood sugar	Remarks
Tungstic acid filtrate—Concluded						
July 30	Rabbit 105		gm	10 30 a.m.	per cent 0 042	Convulsions. 5 cc 25 per cent glucose intravenous. Recovered at once
" 30				1 30 p.m.		Convulsions 5 cc 25 per cent glucose intravenous. Recovered in 10 min
" 30				3 30 "		Convulsions and death
" 27	Rabbit 109.	♂	1,928	2 15 "		17 cc filtrate as used in above experiment.
" 29				12 50 a.m.		Violent convulsions and death
" 30	Rabbit 134.	♀	1,263	4 30 p.m.		20 cc filtrate from tungstic acid precipitated from passage blood
" 31				11 20 "	0 018	Convulsions and death
" 30	Rabbit 132	♀	920	4 45 "		5 cc = 80 cc filtrate as above concentrated by boiling.
Aug. 1				4 45 a.m.	0 010	Convulsions and death
Water extract of autoclaved passage blood.						
July 26	Rabbit 102.	♀	2,384	4 00 p.m.		20 cc. water extract of autoclaved blood = 2 cc. passage blood.
" 27				6 40 a.m.	0 083	
" 27				10 20 p.m.	0 026	Convulsions. 10 cc. 25 per cent glucose subcutaneous. Died at once.
" 28	Rabbit 120.	♀	961	6 00 "		15 cc. = 100 cc. water extract of 5 cc. autoclaved passage blood. Concentrated over a free flame.

## Water extract of autoclaved passage blood—Concluded.

Aug. 1				6 00 a.m.	0 042	Convulsions. Kept alive 28 hrs. more by glucose administration.
" 1				12 00 n.		
Aug. 1	Rabbit 152	♂	1,355	7 30 p.m.		2 cc. air current concentrate water extract = 1 cc. auto-claved passage blood.
" 4				8 45 a.m.	0 032	Collapsed 5 cc. 25 per cent glucose intravenous. Recovered at once.
" 4				10 45 "		Very weak. 5 cc. 25 per cent glucose subcutaneous.
" 4				11 30 "		Convulsions and death.

Precipitation of active principle by (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>.

July 27	Rabbit 116.	♂	1,300	5 30 p.m.		7 cc watery solution of precipitate from passage blood by ammonium sulfate $\frac{1}{3}$ saturation. Equivalent 5 cc. blood.
" 28				5 00 a.m.	0 065	Collapsed 5 cc. 25 per cent glucose subcutaneous. Convulsions
" 28				9 00 p.m.		
" 28				9 30 "		Animal able to sit upright.
" 28				11 45 "		Collapsed. 5 cc. 25 per cent glucose. Died in 15 min.
" 28				12 00 m.	0 060	At death.
" 27	Rabbit 117.	♂	1,230	5 30 p.m.		3 cc. as used above.
" 28				5 00 a.m.	0 087	Convulsions. 5 cc 25 per cent glucose subcutaneous. Recovered.
" 28				8 30 p.m.		



TABLE VII—*Concluded.*

Date	Animal	Sex	Weight	Time	Blood sugar	Remarks
Precipitation of active principle by $(\text{NH}_4)_2\text{SO}_4$ —Concluded						
July 28	Rabbit 117		gm	10 30 p.m.	per cent	Very weak 5 cc. 25 per cent glucose subcutaneous. Partial recovery.
" 28				11 30 "		Convulsions 5 cc 25 per cent glucose subcutaneous.
" 28				11 35 "	0 048	Died at once At death
" 31	Rabbit 126.	♀	1,048	6 00 "		10 cc watery solution of residue after evaporation of 70 per cent alcohol extract of precipitate from passage blood by $\frac{2}{3}$ saturated ammonium sulfate.
Aug. 2				7 30 a.m.		Convulsions 10 cc 25 per cent glucose subcutaneous.
" 2				10 40 "		Recovered Convulsions 5 cc. 25 per cent glucose intravenous.
" 2				2 30 p.m.		Recovered Convulsions. 5 cc 25 per cent glucose subcutaneous.
" 2				10 00 "		Recovered Convulsions 5 cc 25 per cent glucose subcutaneous.
" 3				12 30 a.m.		Recovered Collapsed. 5 cc 25 per cent glucose subcutaneous. No recovery.
" 3				3 30 "	0 010	Died.

As profound hypoglycemia can be produced in normal rabbits by a variety of agents one is led to ask the question—May not all these agencies act in the same fundamental manner? Mann and Magath (4) have shown that hepatized animals develop hypoglycemia and that the symptoms can be antidoted for a time by glucose. It is possible that extensive liver damage, as in coccidial infection in rabbits, might be the underlying cause of certain cases of spontaneous hypoglycemia noted. There is a possibility, and it is only a possibility, that insulin, guanidine, and certain constituents of plant extracts are related chemically in some fundamental manner.

#### SUMMARY.

1. Profound hypoglycemia has been observed to occur in normal rabbits under the following circumstances: (a) Animals injected with certain plant extracts; (b) insulinated rabbits (extreme overdosage); (c) animals injected with guanidine sulfate; (d) spontaneous occurrence (*Coccidia oviforme* found in liver); and (e) after prolonged fasting.

2. The blood of normal rabbits which have developed hypoglycemia under any one of the above circumstances has produced profound hypoglycemia when injected into other normal rabbits.

3. The blood of normal rabbits which have so developed hypoglycemia has likewise produced hypoglycemia when injected into other normal rabbits.

4. Animal passage (rabbit) of a hypoglycemia-producing principle would seem to be possible to an indefinite extent.

5. The symptoms of hypoglycemia which have been observed in these experiments are weakness, which may lead to collapse, convulsions, and death.

6. The symptoms of extreme weakness or convulsions have been relieved by the administration of glucose. The symptoms continued to recur at intervals of a few hours. They were in most instances effectively relieved for a time by glucose, but death has ultimately resulted.

7. The following observations have been made with regard to the nature of the hypoglycemia-producing principle: (a) It is active after autoclaving at 15 pounds pressure; (b) it can be dialyzed; (c) watery solutions can be concentrated by boiling

over a free flame; (d) it is present in the filtrate obtained after treating passage blood with tungstic acid (Folin-Wu technique); and (e) it can be removed from passage blood by ammonium sulfate.

8. A lethal dose for a full grown rabbit may be contained in as small an amount of passage blood as 0.05 cc.

This research has been financed by a grant from the College of Physicians and Surgeons of the Province of Alberta to whom I wish to express my best thanks.

*Addendum*—Recently I have obtained through the kindness of Dr. F. C. Mann of the Mayo Clinic a sample of blood from a hepatized dog. The injection of this blood into normal rabbits has been followed by a state of hypoglycemia

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## **SOME BASIC SUBSTANCES FROM THE JUICE OF THE ALFALFA PLANT.\***

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In a recent paper in this Journal,<sup>1</sup> we stated the distribution of nitrogen, as determined by Hausmann's modified method, in the filtrate obtained from the precipitate produced by adding 53 per cent by weight of alcohol to the undiluted juice of the green alfalfa plant. It was there shown that before hydrolysis with strong acids about 40 per cent of the nitrogen was precipitated by phosphotungstic acid, whereas after boiling with 20 per cent HCl for 14 hours the amount thus precipitated was only half as much.

An attempt to determine the nature of the substances precipitated by phosphotungstic acid before hydrolysis also was described, but no satisfactory evidence of the presence of either arginine, histidine, or lysine was obtained. The amount of substance taken for this experiment, however, was too small to permit of final conclusions.

In the present paper we give the results of an examination of a much larger quantity of the alfalfa juice. The filtrate from the precipitate produced by adding 53 per cent by weight of alcohol to 3,415 cc. of the juice of the alfalfa plant was concentrated, boiled with 25 per cent sulfuric acid for 12 hours, and the basic products of hydrolysis were precipitated with phosphotungstic acid. By employing Kossel's method for determining the basic amino-acids produced by the hydrolysis of proteins we found that the silver-

\* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

<sup>1</sup> Osborne, T. B., Wakeman, A. J., and Leavenworth, C. S., *J. Biol. Chem.*, 1922, lxi, 411.

baryta precipitate yielded arginine, containing nitrogen equal to 3.6 per cent of the nitrogen present in the filtrate from the precipitate from the 53 per cent alcohol precipitate. This was identified as the picrolonate.

Another substance yielding a crystalline hydrochloride, which did not give the reaction with diazobenzenesulfonic acid characteristic of histidine, formed most of the precipitate produced by  $\text{HgSO}_4$ . When converted into the picrate this melted sharply at  $298^\circ$ . If histidine is present among the bases its proportion must be very small. The amount of the above substance was not enough to permit of its identification. It contained nitrogen equal to 2.2 per cent of the total nitrogen of the original solution. Since 90 per cent of the nitrogen of the second silver-baryta precipitate was recovered in the arginine picrolonate it is evident that besides the unidentified base obtained from the  $\text{HgSO}_4$  precipitate and arginine there is little, if anything else among the products of hydrolysis thus precipitable by silver.

The filtrate from the silver-baryta precipitate, which should contain lysine, was found to contain stachydrine also in much larger proportion than lysine. The nitrogen in the stachydrine was equal to 5.4 per cent of the nitrogen in the filtrate from the 53 per cent alcohol precipitate and that of the lysine to 1.16 per cent. These bases were separated by means of  $\text{HgCl}_2$ , the fraction obtained at acid reaction consisting almost wholly of the mercury salt of stachydrine, while that precipitated at alkaline reaction contained the mercury compound of lysine. Both of these substances were identified as picrates.

The presence of stachydrine thus established confirms Steenbock's earlier discovery<sup>2</sup> of this betaine among the constituents extracted by water from alfalfa hay. Whether all the stachydrine found by us exists in the free state in the alfalfa juice is not demonstrated by our experiments. Steenbock's work indicates that at least a part of this base is free, unless all that he isolated was liberated by enzymes during the curing of the hay or its extraction by water.

The fact that practically all the nitrogen precipitated by  $\text{HgCl}_2$  at slight acid reaction was identified as stachydrine shows that significant quantities of other bases thus precipitable were not present.

<sup>2</sup> Steenbock, H., *J. Biol. Chem.*, 1918, xxxv, 1.

## EXPERIMENTAL.

The filtrate from the precipitate produced by adding 53 per cent by weight of alcohol to 3,415 cc. of the fresh, clear, undiluted juice of the alfalfa plant was concentrated to 1,000 cc. and found to contain 8.26 gm. of nitrogen. Of this 3.15 per cent was ammonia nitrogen and 33.3 per cent amino nitrogen as determined by Van Slyke's method. The total solids were 155 gm. To the remaining solution were added 250 gm of  $\text{H}_2\text{SO}_4$  and the mixture was boiled for 12 hours under a reflux condenser.

The humin which separated was removed by centrifuging and washed with water. This contained 0.77 gm. of nitrogen<sup>3</sup> or 9.33 per cent of the nitrogen of the juice. The solution then contained 7.39 gm of nitrogen of which 1.171 gm were ammonia nitrogen and 3.621 gm were amino nitrogen, equal, respectively, to 14.18 and 43.83 per cent of the nitrogen of the juice. Enough  $\text{Ba}(\text{OH})_2$  was added to remove 1.25 gm of  $\text{H}_2\text{SO}_4$  and the filtrate and washings from the  $\text{BaSO}_4$  were concentrated to 2,500 cc., thus making the solution contain 5 per cent of  $\text{H}_2\text{SO}_4$ . The solution then contained 7.37 gm of nitrogen, thus showing that there was little loss of nitrogen in the  $\text{BaSO}_4$  precipitate. A 20 per cent solution of phosphotungstic acid in 5 per cent  $\text{H}_2\text{SO}_4$  was added as long as a precipitate formed, which after standing a few hours was filtered out and washed with 2.5 per cent phosphotungstic acid in 5 per cent  $\text{H}_2\text{SO}_4$ .

This precipitate of the bases was decomposed with  $\text{Ba}(\text{OH})_2$ , the excess of Ba was converted into carbonate, and the solution was heated therewith until ammonia was removed. After filtering,  $\text{Ag}_2\text{SO}_4$  and  $\text{Ba}(\text{OH})_2$  were added until an excess of Ag was demonstrated in the filtrate. The precipitate was then filtered out, washed with saturated  $\text{Ba}(\text{OH})_2$  solution, the silver salts were suspended in dilute  $\text{H}_2\text{SO}_4$  and decomposed with  $\text{H}_2\text{S}$ . The filtrate and washings from the  $\text{Ag}_2\text{S}$  were concentrated and  $\text{HgSO}_4$ , dissolved in 5 per cent  $\text{H}_2\text{SO}_4$ , was added as long as a precipitate formed. After standing overnight the precipitate was washed thoroughly with water containing some of the  $\text{HgSO}_4$  reagent and decomposed with  $\text{H}_2\text{S}$ , and the  $\text{HgS}$  washed. The filtrate and washings were treated with  $\text{Ba}(\text{OH})_2$  to remove  $\text{H}_2\text{SO}_4$ , and the excess of Ba was removed as carbonate. The solution was then concentrated to 500 cc. and found to contain 0.19 gm of nitrogen, equal to 2.3 per cent of the original nitrogen.

This solution was evaporated, the residue dissolved in dilute  $\text{HCl}$ , decolorized with norit, concentrated on the steam bath to small volume, and allowed to crystallize by slow concentration over  $\text{CaO}$ . Finely developed, flat prisms separated which, under the microscope, appeared to be perfectly pure. After evaporating to dryness the residue was dissolved in a little hot water and alcoholic picric acid added as long as a precipitate formed. This was filtered out, washed with alcohol, recrystallized from hot water, and 0.16 gm. of a picrate obtained which crystallized in long

<sup>3</sup> These and subsequent figures are corrected for the portions taken for analysis during the progress of this investigation.

silky needles, melting sharply at  $298^{\circ}$ . The mother liquor from these crystals, when evaporated to dryness, left a residue which, when recrystallized from water, yielded 0.07 gm. of crystals of the same form and melting point as the main crop. By extracting products of hydrolysis obtained in a former experiment by boiling with 5 per cent HCl, extracting with butyl alcohol, and then shaking out the latter with water, a picrate, having the same crystalline form and melting point, was obtained.

Although derived from the fraction which, by Kossel's method, should yield histidine, the properties of this picrate are such as to show that it is not histidine picrate. This is also shown by the absence of the color reaction with diazobenzenesulfonic acid.

The united fractions of the above described picrate were decomposed by acidifying with HCl and extracting the picric acid with ether.

On treating the aqueous layer with silver sulfate the precipitated silver salts were found to be only partly soluble in ammonia. The soluble part contained very little nitrogen other than ammonia and consisted chiefly of silver chloride. On the other hand, the insoluble part when washed free from ammonia and digested with strong hydrochloric acid yielded a solution from which crystals separated in well formed, long plates. These melted, not sharply, at about  $252^{\circ}$  and contained 31.8 per cent of nitrogen. Although the above data indicate the presence of a purine base, the identity of this substance with any one of the known members of this group has not yet been established.

The filtrate from the  $\text{HgSO}_4$  precipitate was freed from Hg by  $\text{H}_2\text{S}$  and from  $\text{H}_2\text{SO}_4$  by  $\text{Ba}(\text{OH})_2$ . The solution was acidified with  $\text{HNO}_3$  and treated with  $\text{AgNO}_3$  and baryta as usual in applying Kossel's method. The precipitate thus produced was decomposed with  $\text{H}_2\text{S}$  and found to contain 0.30 gm. of nitrogen, or 3.6 per cent of the total nitrogen in the original solution. An attempt to convert this base into the picrate failed to yield a crystallizable product. Picric acid was therefore removed and the picrolonate obtained. This melted at  $234^{\circ}$ , weighed 1.8 gm., and, when mixed with pure arginine picrolonate obtained from protein, the melting point was unchanged. A second crop, which melted at  $227^{\circ}$ , weighed 0.35 gm.

The arginine picrolonate contained 0.82 gm. of arginine, whereas the total nitrogen in the second silver-baryta precipitate was equivalent to 0.91 gm. It thus appears that practically all this fraction of the bases is arginine and that this amino-acid constitutes a very small proportion of the nitrogenous constituents of the alfalfa juice.

The filtrate from the silver-baryta precipitate when freed from Ba with  $\text{H}_2\text{SO}_4$  was concentrated and 8.7 gm. of  $\text{K}_2\text{SO}_4$  were removed by crystallization from dilute alcohol. The filtrate and washings were concentrated to 80 cc., 5 per cent of  $\text{H}_2\text{SO}_4$  was added, and the bases were precipitated with phosphotungstic acid. This precipitate was decomposed with  $\text{Ba}(\text{OH})_2$ , the excess of Ba removed by  $\text{CO}_2$ , the solution concentrated to 200 cc., and nitrogen, determined in 5 cc., found to be equal to 0.768 gm. The remaining 195 cc. were treated with dilute HCl, containing one molecule

of HCl for each atom of N, concentrated *in vacuo* to 200 cc., and  $\text{HgCl}_2$ , dissolved in alcohol, was added.

The precipitate which first formed redissolved until a considerable quantity of  $\text{HgCl}_2$  had been added. After standing overnight the precipitate was filtered out and washed with alcohol of about the same concentration as the filtrate. The heavy crystalline precipitate was twice recrystallized from hot water and decomposed with  $\text{H}_2\text{S}$ . The filtrate from the  $\text{HgS}$  was concentrated and five successive crops of crystals were obtained, each of which melted at  $235\text{--}236^\circ$ , thus indicating stachydrine chloride which melts at this temperature.

These fractions were united and found to contain 0.45 gm. of nitrogen as calculated from an aliquot part of the 250 cc of this solution. This is equal to 5.4 per cent of the nitrogen of the original solution, or to 58.5 per cent of the fraction of the bases which should contain the lysine according to Kossel's method for determining bases in the products of protein hydrolysis. The remaining 240 cc of the solution were freed from HCl with  $\text{Ag}_2\text{CO}_3$ , the filtrate and washings from the  $\text{AgCl}$  concentrated to about 200 cc, and one molecule of picric acid for each atom of nitrogen was added. The solution was then concentrated to crystallization. This picrate melted at  $196^\circ$  when twice recrystallized, thus agreeing in melting point with stachydrine picrate.

The total stachydrine picrate recovered from this solution weighed 10.53 gm, equal to 4.34 gm of stachydrine, allowing for the aliquots removed for nitrogen determinations. When once recrystallized this melted at  $196^\circ$  and contained 14.90 per cent of nitrogen, calculated for stachydrine picrate 15.05 per cent. The amount of stachydrine calculated from the nitrogen precipitated by Hg is 4.59 gm, hence there is no doubt that this is the only base thus precipitated by  $\text{HgCl}_2$ .

The filtrate from the mercuric chloride precipitate of stachydrine was concentrated to about 150 cc and made alkaline with  $\text{Ba(OH)}_2$ . The precipitate was washed with dilute baryta water, dissolved in hot water, acidified with HCl, and decomposed with  $\text{H}_2\text{S}$ . The filtrate from the  $\text{HgS}$  was freed from Ba with  $\text{H}_2\text{SO}_4$ , concentrated to small volume on the water bath, and then over CaO to a syrup. Since no crystals separated the syrup was dissolved in water, 5 per cent of  $\text{H}_2\text{SO}_4$  added, and then phosphotungstic acid was added as long as a precipitate formed. The precipitate was decomposed with  $\text{Ba(OH)}_2$ , the excess of Ba removed by  $\text{CO}_2$ , and the filtrate and washings were concentrated to 100 cc. Nitrogen was then determined in 5 cc. to be equal to 0.160 gm in the whole solution.

The remainder was concentrated to small volume, alcohol added, and then 1.2 gm of picric acid were added in alcoholic solution. The picrate thus precipitated was filtered out and recrystallized from water. This weighed 1.17 gm, equal to 1.28 gm, allowing for the known solubility of lysine picrate in the mother liquor. The crystals, which resembled pure lysine picrate when recrystallized, contained 18.56 per cent of nitrogen; calculated for lysine picrate 18.67 per cent. This melted with decomposition simultaneously with a preparation of lysine picrate obtained from



protein. The total lysine picrate thus estimated is equal to 0.499 gm. of lysine containing 0.095 gm. of nitrogen, or to 1.16 per cent of the original nitrogen.

The mercury precipitate of the lysine contained 0.160 gm. of nitrogen, which if all were lysine would be equal to 0.833 gm. of lysine. The lysine recovered as picrate was 0.499 gm, leaving 0.334 gm. unidentified. A part of this difference is probably due to an incomplete recovery of all of the lysine as picrate, but the possibility is not excluded that a small amount of some other base may have been present in the mercury precipitate.

The filtrate from the alkaline mercury precipitate was freed from Hg and  $\text{SO}_4$  with  $\text{Ba}(\text{OH})_2$ , the Ba quantitatively removed with  $\text{H}_2\text{SO}_4$ , and the solution concentrated to a syrup, but no crystalline separation could be secured. This was then dissolved in 5 per cent  $\text{H}_2\text{SO}_4$ , precipitated with phosphotungstic acid, the precipitate decomposed with  $\text{Ba}(\text{OH})_2$ , and the excess of Ba removed as carbonate. The solution was made up to 100 cc and nitrogen determined in 5 cc. to be equal to 0.0738 gm in the whole solution. To the remaining solution 1.12 gm of picric acid were added, but no satisfactory crystalline picrate was obtained.

Whether or not these bases exist free in the alfalfa juice remains to be determined. The fact that after complete hydrolysis with strong acids the proportion of nitrogen precipitable by phosphotungstic acid is reduced to about one-half and that of the free amino nitrogen increased by about 50 per cent, shows that much of the nitrogen of the juice occurs in more complex combinations than free amino-acids and betaines.

We have found that about 75 per cent of the total nitrogen of the juice not precipitated by 53 per cent alcohol can be precipitated by mercuric acetate and sodium carbonate. It is hoped that the examination of this precipitate, which is now in progress, may shed some light on the nature of the nitrogenous constituents of the alfalfa juice.

The results of this investigation show that the method devised by Van Slyke for analyzing the products of protein hydrolysis cannot be used for analyzing the water-soluble constituents of forage plants and that such analyses as have been published have little if any value.

## COMPARATIVE METABOLISM OF CERTAIN AROMATIC ACIDS.

### V. FATE OF SOME RING SUBSTITUTION PRODUCTS OF PHENYLACETIC ACID IN THE ORGANISMS OF THE DOG, RABBIT, AND MAN.

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Some time ago it was shown that when phenylacetic acid is ingested by a human being an entirely different mode of detoxication is adopted than when the same substance is fed to lower animals (1). Later it was found that the glutamine which is employed as the detoxicating agent by the human organism is actually synthesized by the body, and this in relatively large amounts, the nitrogen for the amide and amino groups being taken from that fraction of the total nitrogen which would otherwise appear in the urine as urea (2). In this way the urea nitrogen could be reduced from 60 to 20 per cent of the total 24 hour output. Furthermore, it was noted that the glutamine could be synthesized not only on a protein diet, but just as easily and efficiently on a mixed carbohydrate-fat diet or even during a period of fasting. Thereupon, two very interesting questions proposed themselves: (1) Where does this glutamine synthesis take place? (2) Can this synthesis be used as an organic function test?

An attempted solution of one or perhaps both of these questions formed the *ultimate* object of the present work. A preliminary and more fundamental matter, however, had to be settled first; the discovery, namely, of a suitable quantitative method for the determination of the amount of phenylacetyl glutamine in the urine after the ingestion of phenylacetic acid, for the present means of extracting the compound from the evaporated urine

with ethyl acetate is not only tedious but is at best merely qualitative. Of two methods which at first seemed applicable, both proved unsatisfactory. The first had as its basis a determination of the amide nitrogen of the glutamine, but the continual presence of urea in the urine made such a determination impracticable. In the second method which rested on the determination of the amino nitrogen after the hydrolysis of the compound into phenylacetic acid and glutamine, the hydrolysis gave rise to quantities of glycocoll from varying amounts of hippuric acid ever present in normal human urine, which, of course, interfered seriously with the determination; while on the other hand, the formation of corresponding amounts of benzoic acid made a quantitative estimation even of the phenylacetic acid quite an impossibility.

In an attempt, therefore, to overcome these difficulties we planned to administer to human subjects a number of *ring derivatives* of phenylacetic acid, in the hope that we might thus obtain some corresponding *derivative* of phenylacetyl glutamine which could be isolated quantitatively from the urine. This was the primary and immediate end we had in view. As of secondary interest we decided to feed the same substances also to several of the lower animals, especially to dogs and rabbits, in order to study their comparative metabolism.

The following compounds were employed: *o*-nitrophenylacetic acid, *o*-aminophenylacetic acid, *o*-hydroxyphenylacetic acid, *o*-chlorophenylacetic acid, and 2-4-dinitrophenylacetic acid. We had shown previously that *p*-nitrophenylacetic acid (3) and *p*-hydroxyphenylacetic acid (4) pass through the human organism unchanged, but that they are in part conjugated in the organisms of the lower animals. Thus a hen excretes *p*-nitrophenylacetic acid as *p*-nitrophenacetornithuric acid and a dog excretes it in part as *p*-nitrophenaceturic acid. In the course of the present series of investigations we were surprised to find that only one of the five substances used, namely the *o*-chloro compound, is conjugated by the human body, and still more peculiarly that the detoxicating agent is not glutamine as one would most likely expect but glycocoll, so that in this particular the human organism acts exactly like the dog and excretes the *o*-chlorophenylacetic acid as *o*-chlorophenaceturic acid.

## EXPERIMENTAL.

In general the method used in the work was the following. The substance to be fed was dissolved in water, or if insoluble, was neutralized with dilute sodium hydroxide and administered as a water solution of the sodium salt. In the case of the lower animals the fairly dilute solution was introduced directly into the stomach by means of a stomach tube. The bladder was then emptied by gentle pressure and the subsequent urine collected for a period of 36 or 48 hours. The subjects were carefully watched for clinical symptoms resulting from the ingested substance. The volume of each collection of urine was measured, the physical constants were noted, and tests were made for albumin and sugar. The urine was then neutralized, evaporated on a water bath to a thick syrup, cooled, acidified to Congo red with a mineral acid, and finally extracted for 6, 9, or 12 hours with ethyl acetate in a rotary extracting apparatus. The ethyl acetate was then evaporated to dryness by stages, and in case no crystals appeared, the residue was dissolved in boiling water and boiled once or twice with animal charcoal. The various crops of crystals were then collected and recrystallized until the constancy of the melting point showed that a substance of a fairly high grade of purity had been obtained.

*1 o-Nitrophenylacetic Acid.*

*o*-Nitrophenylacetic acid (M. P.  $141^{\circ}\text{C}$ .) was fed to a rabbit in 1 gm. doses until a total of 3 gm. had been fed. The substance was apparently entirely harmless, for the animal showed no signs of discomfort. From the urine the material was recovered unchanged. As it is fairly insoluble in water, it separated readily in well formed crystals, which after a single recrystallization from water melted at  $139\text{--}141^{\circ}\text{C}$ . We were able to feed a dog of 32 kilos body weight two doses of 7 gm. each within a period of 25 hours. A man received two doses of 5 gm. each within a period of 36 hours and complained of no uneasiness whatever. We also fed a hen two doses of 1 gm. each, hoping to be able to isolate a new ornithine compound. As in the case of the rabbit, so in each of the other cases was the substance recovered unchanged. The amounts isolated from the various feedings will be found in Table I.

*2. o-Aminophenylacetic Acid.*

This compound was prepared according to the method of Neber (5), by the reduction of *o*-nitrophenylacetic acid with ferrous sulfate in a barium hydroxide solution. The barium salt of the *o*-aminophenylacetic acid thus formed was then treated with dilute sulfuric acid. The resulting product had a melting point of 118-119°C., but was mixed with leafy crystals melting somewhat higher. These were apparently the inner anhydride of the compound; namely, oxyindole. To remove this impurity the barium salt of the acid was converted into the soluble sodium salt by boiling a solution of the former with the calculated amount of anhydrous sodium carbonate. The barium carbonate formed during the reaction was filtered off, and the resulting water solution of the sodium salt used for feeding. It was given to human beings in 2 gm. doses without causing any ill effects. Dogs received the substance in 4 gm. doses, and rabbits in doses of 1 gm. each.

The urines, which were normal as to color, deepened in color on evaporation. The ethyl acetate extract of the acidified residue of the evaporated urines was purple in color, due no doubt to the action of the mineral acid upon the *o*-aminophenylacetic acid, converting it into oxyindole through the loss of a molecule of water. Only oxyindole (M. P. 122-126°C.), and this in small amounts, was isolated from the urines of man and dogs, but an acetylated product was extracted from the urine of rabbits. This product was most likely excreted as the acetylated *o*-aminophenylacetic acid, which was later dehydrated either through contact with the mineral acid or by merely standing in the air. At any rate, the compound, which was obtained only in small amounts, reacted exactly like the synthetic acetyloxyindole which we prepared, in that the percentages of nitrogen of the two products corresponded quite closely, and both preparations split off acetic acid when heated with sulfuric or hydrochloric acids, as could be detected by the odor.

The acetyloxyindole was synthesized as follows: 2 gm. of *o*-aminophenylacetic acid (dry) were heated to boiling under a reflux condenser for 3 minutes with an excess (12 cc.) of acetic anhydride. *o*-Acetylaminophenylacetic acid is first formed, but

is immediately dehydrated by the hot acetic anhydride. Thus the acetyloxyindole is formed in one operation. The compound is then poured into water, from which it crystallizes out as long, white, hair-like needles (M. P. 124–126°C.).

Nitrogen determinations according to the Kjeldahl method on both samples resulted as follows:

Acetyloxyindole, synthetic . . . . .	7	94	per cent N.
“ extracted from urine of rabbits	8	19	“ “ “
Calculated percentage in acetyloxyindole .	8	00	“ “ “

The oxyindole recovered from the urines of the human being and the dog was without doubt a secondary product formed outside the body by a simple process of dehydration. The acetylation of the oxyindole by the rabbit is an addition to the number of acetylation reactions and is especially interesting since this type of reaction is none too frequent.

### 3 *o*-Hydroxyphenylacetic Acid.

As this compound cannot be made in the usual way, *i.e.* by treating *o*-aminophenylacetic acid with nitrous acid, an indirect method had to be used; namely, the reduction of *o*-hydroxymandelic acid with hydriodic acid.

To effect the synthesis, therefore, *o*-hydroxybenzaldehyde (Eastman) was treated with potassium cyanide and hydrochloric acid according to the method of Ploschl (6). In this way a very poor yield of *o*-hydroxymandelic acid was obtained. This substance was then reduced with hydriodic acid according to the method of Baeyer and Fritsch (7) and thereby converted into *o*-hydroxyphenylacetic acid with a melting point of 135–137.5°C.

Like the other ortho derivatives of phenylacetic acid the *o*-hydroxy compound proved to be quite harmless physiologically so that it could be ingested in relatively large doses. Like the other compounds, too, it was excreted in the urine unchanged. A rabbit was fed the substance first in 0.5 gm., then in 1 gm. doses; a man received two doses of 2 gm. each, and a dog received as much as 4 gm. at one dose without showing the slightest signs of intoxication. As the compound gives a positive Millon's reaction the course of its elimination from the body could be followed with

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considerable precision. It was interesting to note, therefore, that after a dose of 2 gm. most of the substance was eliminated from the body within 5 or 6 hours.

### 4. *o*-Chlorophenylacetic Acid.

*o*-Chlorophenylacetic acid was made according to the method of Mehner (8) as follows: 75 gm. of potassium cyanide were dissolved in the least possible amount of hot water. This syrupy mass was then placed in a flask fitted with a reflux condenser and to it were added 150 gm. of *o*-chlorobenzyl chloride, with sufficient alcohol to insure an intimate mixing of the two layers. The mixture was then boiled for a period of at least 5 hours. (A longer period is often necessary.) On cooling, a mass of potassium chloride was deposited on the bottom of the flask. This was washed well with alcohol after the liquid contents of the flask had been poured off, and the washings were added to the rest of the liquid. The alcohol was then distilled off from the liquid mixture, leaving an oily residue. This oil was then extracted repeatedly with ether, dried with calcium chloride, and redistilled. The *o*-chlorophenyl cyanide thus obtained can be distilled between 235 and 250°C. *o*-Chlorophenyl acetamide was then made by dissolving the *o*-chlorophenyl cyanide in concentrated sulfuric acid and diluting this solution with 3 volumes of water. After standing  $\frac{1}{2}$  hour the solution was poured into 30 volumes of cold water, whereupon the *o*-chlorophenyl acetamide crystallized out in the form of white, leafy crystals (M. P. 172–175°C). *o*-Chlorophenylacetic acid was prepared by dissolving the *o*-chlorophenyl acetamide in boiling water and adding to this solution enough sulfuric acid to make the whole strongly acid to Congo red. This solution was kept boiling while a dilute solution of sodium nitrite was added drop by drop until the reaction was completed. On cooling, the *o*-chlorophenylacetic acid crystallized out in the form of long, white needles. After recrystallization from hot water the substance was found to melt at 95°C.

The *o*-chlorophenylacetic acid was fed to a middle sized dog in doses of 3 gm. Two such doses were given. The substance seemed to cause no discomfort to the animal. Upon working up the urine in the usual way, 0.3 gm. of the acid was recovered unchanged, while 1.3 gm. were found in combination with glycocholic acid;

*i.e.*, as *o*-chlorophenaceturic acid. This was an extremely poor yield. After a human being had ingested the same amount of the *o*-chlorophenylacetic acid, 0.6 gm. of the acid was recovered unchanged, while 1.20 to 1.25 gm. of *o*-chlorophenaceturic acid were extracted from the urine. After feeding the *o*-chlorophenylacetic acid to rabbits or after injecting it intravenously, only small amounts of the unchanged acid could be isolated from the urine, and no conjugation product could be found.

The *o*-chlorophenaceturic acid obtained from the urine of dogs and human beings is a colorless compound, quite soluble in hot water, from which it crystallizes in the form of irregular needles. After several recrystallizations from hot water the melting point was found to be 130.5–133°C. Upon analysis the substance yielded the following results.

0.2011 gm substance yielded 0.3880 gm CO <sub>2</sub> and 0.0801 gm H <sub>2</sub> O
Calculated C 52.63, H 4.38, N (Kjeldahl) 6.11 per cent.
Found. " 52.54, " 4.41, " ( " ) 6.24 " "

#### *Synthesis of o-Chlorophenaceturic Acid.*

For the synthesis of this compound it was necessary first to prepare the *o*-chlorophenylacetyl chloride. Accordingly, 3 gm. of carefully dried *o*-chlorophenylacetic acid (M. P. 95°C.) were mixed in a distillation flask with 3.6 gm. of phosphorus pentachloride. The reaction took place at room temperature, resulting in a liquid mixture. When the reaction was finished the phosphorus oxychloride was distilled off, and then at a reduced pressure of 12 mm. and at a temperature of 119–121°C. a colorless, oily liquid distilled over. This was found to be quite pure *o*-chlorophenylacetyl chloride.

To synthesize the *o*-chlorophenaceturic acid, 0.7 gm. of glycocholic acid was dissolved in 3.5 cc. of water, and the solution was made alkaline with sodium hydroxide. Then one and a half times the theoretical amount of *o*-chlorophenylacetyl chloride (2.7 gm.) was added, a few drops at a time. After each addition of the acid chloride the mixture was thoroughly shaken and tested with litmus. As soon as the mixture became acid in reaction a small amount of alkali was added to insure a slightly alkaline medium. After all the acid chloride had been added the mixture was made



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strongly acid with sulfuric acid. A white precipitate at once appeared, consisting of *o*-chlorophenylacetic acid and *o*-chlorophenaceturic acid. This was filtered by suction and washed several times with ether to remove the *o*-chlorophenylacetic acid. The *o*-chlorophenaceturic acid was then recrystallized several times from hot water and thoroughly dried. It then melted quite sharply between 134 and 135°C. The yield was about 35 per cent of the theoretical.

In a second attempt at the synthesis, 4 gm. of *o*-chlorophenylacetic acid were treated with 4.9 gm. of phosphorus pentachloride. From this we obtained 2.7 gm. of *o*-chlorophenylacetyl chloride, which distilled over at a temperature of 118–120°C. under a pressure of 10 mm. of mercury. This 2.7 gm. of the acid chloride were then treated with 1.1 gm. of glycocoll according to the method described above. A yield of 1 gm. of *o*-chlorophenaceturic acid, melting at 135°C., was obtained. After drying to constant weight the substance showed the following analysis.

0.1703 gm. substance yielded	0.3291 gm. CO <sub>2</sub> and	0.0689 gm. H <sub>2</sub> O
0.1290 " " "	6.90 cc. nitrogen at 755 mm. pressure and	
19°C		

Calculated for C <sub>10</sub> H <sub>10</sub> O <sub>3</sub> NCI	C 52.63, H 4.38, N 6.07 per cent
Found.	" 52.70, " 4.49, " 6.11 " "

The synthetic substance was identical with the *o*-chlorophenaceturic acid obtained from the urine of man and dogs after the feeding of *o*-chlorophenylacetic acid.

*o*-Chlorophenaceturic acid is practically insoluble in benzene, petroleum ether, and absolute ether. It is moderately soluble in chloroform and ethyl acetate, but dissolves readily in acetone and alcohol.

### 5. 2-4-Dinitrophenylacetic Acid.

This compound was prepared according to the method of Borsche (9) by treating phenylacetic acid with a mixture of 2 parts of sulfuric acid and 1 part of nitric acid. The melting point of the product varied between 178 and 181°C. Upon feeding the substance, we found it to be as harmless as either the *o*-nitrophenylacetic acid or the *p*-nitrophenylacetic acid. A man ingested 2, 3, and 5 gm. of the acid in three respective doses. After the ingestion

of the 10 gm., 3.7 gm. of the unaltered compound were isolated from the urine. After two doses of 4 and 5 gm., respectively, to a middle sized dog, 4.42 gm. were recovered unchanged from the urine. Two rabbits received together a total of 5 gm. in 1 gm. doses. Here again, 2.35 gm. of the substance were recovered as the unchanged acid.

Table I summarizes the data of the comparative metabolism on the different compounds when man and the lower animals were used as experimental subjects.

TABLE I

Substance	Fed			Acetylated			Combined with glycocoll			Excreted free.		
	Man	Dog	Rabbit	Man	Dog	Rabbit	Man	Dog	Rabbit	Man	Dog	Rabbit
	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm
<i>o</i> -Nitrophenylacetic acid	10	14	2	0	0	0	0	0	0	4	7	0
<i>o</i> -Aminophenylacetic acid.	6	6	6	0	0	0	9	0	0	1	35	1
<i>o</i> -Hydroxyphenylacetic acid	8	7	4	0	0	0	0	0	0	3	2	3
<i>o</i> -Chlorophenylacetic acid	12	6	3	0	0	0	3	2	4	0	6	0
2-4-Dinitrophenylacetic acid	10	9	5	0	0	0	0	0	0	3	7	4
											42	2
												35

## SUMMARY.

1. In the present work, which was undertaken primarily in an attempt to discover a derivative of phenylacetyl glutamine which could be isolated quantitatively from human urine, the following derivatives of phenylacetic acid were prepared and fed to human beings, dogs, and rabbits: *o*-nitrophenylacetic acid, *o*-aminophenylacetic acid, *o*-hydroxyphenylacetic acid, *o*-chlorophenylacetic acid, and 2-4-dinitrophenylacetic acid.

2. In connection with *o*-chlorophenylacetic acid, we also prepared *o*-chlorophenaceturic acid and its antecedent *o*-chlorophenylacetyl chloride, neither of which had been synthesized previously. Accordingly, these two substances were studied in somewhat greater detail.

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3. All the above mentioned five compounds were found to be quite non-toxic physiologically. In fact, only two of them, namely *o*-aminophenylacetic acid and *o*-chlorophenylacetic acid, were subjected to detoxication at all—the others passing through the organisms unchanged.

4. This furnishes proof that ortho compounds, contrary to the opinion of some, are not easily oxidized in the organism, and, furthermore, that they are not necessarily more toxic than their isomeric meta and para derivatives. In fact, these ortho compounds were found to be less toxic than phenylacetic acid itself.

5. Of the two compounds which were subjected to detoxication, *o*-aminophenylacetic acid was conjugated only by the rabbit, and this by the very unusual reaction of acetylation, adding thereby a new example to the few cases of this reaction already known. On the other hand, the *o*-chlorophenylacetic acid was excreted unchanged by the rabbit, but was conjugated with glycocoll by the dog and human being. This latter fact was indeed surprising, in that one would have expected the human organism to use glutamine instead of glycocoll as the detoxicating agent.

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# OXIDATION OF GLUCOSE BY IODINE IN THE PRESENCE OF INSULIN.

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## 1. Purpose of this Investigation.

This investigation was undertaken with the purpose of determining whether insulin, alone or in the presence of certain animal fluids, has any influence upon glucose *in vitro*. The establishment of such an influence might have much significance in relation both to the study of carbohydrate metabolism and to the development of methods of assaying insulin.

For this purpose it seemed desirable to use a property for determining the glucose content which is highly specific for this substance and would not be likely to be shown in at all the same degree by products that might result from a change occurring under the influence of the insulin. The rate of oxidation of glucose ( $C_6H_{12}O_6$ ) to gluconic acid ( $C_6H_{12}O_7$ ) by iodine in nearly neutral solution seemed to be an especially suitable property; preliminary experiments on the oxidation of glucose, mannose, fructose, and sucrose by iodine having confirmed the results of previous investigators that the rates are widely different for these different compounds. A series of experiments was therefore made on the comparative rates of this oxidation, using in one case a pure glucose solution, and in other cases glucose solutions which had been previously treated with aqueous insulin extract, alone or mixed with liver extract, blood serum, or oxalated blood.

This investigation forms part of a series of researches on the chemical nature and behavior of insulin undertaken in this laboratory under the general direction of Prof. A. A. Noyes, to whom we desire to express our thanks for advice as to the work and for his aid in the preparation of it for publication. This investigation was

assisted on the financial side from the funds which have been made available by Dr. Bertnard Smith for the general prosecution of insulin researches in this laboratory. We are also indebted to Dr. Smith, and to his associate Dr. Howard West, for cordial cooperation and assistance on the biological sides of this investigation; also to Mr. Albert L. Raymond of this laboratory for many valuable suggestions.

## 2. Previous Researches.

Various investigators have already studied the reaction between sugars and iodine, with reference to the development of methods of analysis. Thus, Romijn<sup>1</sup> early showed that glucose is quantitatively oxidized by iodine in alkaline solution to gluconic acid. He found that aldoses, in general, are oxidized under these conditions, while ketoses remain unchanged; and he devised a method of sugar analysis based on this principle. As the results obtained with potassium or sodium hydroxide were rather irregular, he substituted sodium borate for the alkali. The reaction, although slow, proved to be quantitative in this weakly alkaline solution. Bougault<sup>2</sup> substituted sodium carbonate for the borate; and this gave a much more rapid oxidation and led to a satisfactory method of analysis. Willstatter and Schudel<sup>3</sup> found that the reaction proceeded smoothly to completion when 0.1 N solutions of iodine and sodium hydroxide in the proportion of 2:3 were used. Colin and Liévin<sup>4</sup> modified Bougault's procedure by using disodium hydrogen phosphate in the place of the carbonate. Cajori<sup>5</sup> has recently published an article in which he gives a method of separately determining glucose, fructose, sucrose, and maltose, in mixtures; this method being based on the different behaviors of these sugars towards iodine and towards cupric hydroxide.

The effect of insulin on glucose has been studied by Winter and Smith.<sup>6</sup> These investigators made a series of experiments

<sup>1</sup> Romijn, G., *Z. anal. Chem.*, 1897, xxxvi, 349.

<sup>2</sup> Bougault, J., *J. pharm. et chim*, 1917, xvi, series 7, 97; *Compt. rend. Acad.*, 1917, clxiv, 1008

<sup>3</sup> Willstatter, R., and Schudel, G., *Ber. chem. Ges*, 1918, li, 780.

<sup>4</sup> Colin, H., and Liévin, O., *Bull. Soc. chim.*, 1918, xxiii, series 4, 403.

<sup>5</sup> Cajori, F. A., *J. Biol. Chem.*, 1922, liv, 617.

<sup>6</sup> Winter, L. B., and Smith, W., *Brit. Med. J.*, 1923, i, 12; *cf. J. Physiol.*, 1922-23, lvii, 100.

upon the change in optical rotation produced in glucose and fructose solutions by the addition of small amounts of insulin and liver extracts. They thought they detected, in the case of both sugars, an appreciable change, which reached a maximum in 2 to 4 days and was greatly increased by the addition of phosphate solution. They suggested that the function of insulin might be the activation of the enzyme (presumably present in the liver) which is responsible for the transformation of ordinary  $\alpha$   $\beta$ -glucose into the  $\gamma$  form. The  $\gamma$  sugars are known to be very reactive chemically: in summarizing their properties, Hewitt<sup>7</sup> notes their great activity toward oxidizing agents and their marked instability in the presence of acid and alkali.

### 3. *Experimental Procedure and Materials.*

The oxidations were carried out in a nearly neutral solution with an excess of iodine, so as to insure a nearly complete conversion of glucose into gluconic acid in a convenient period of time. To give the proper acidity, solutions 0.3 molal in  $\text{NaHCO}_3$  or 0.15 molal in  $\text{Na}_2\text{HPO}_4$  were used. In these solutions the reaction is practically complete at room temperature in  $2\frac{1}{2}$  hours. About five times the theoretical amount of iodine was employed. The oxidations were carried on simultaneously in 100 cc. rubber stoppered conical flasks. Since the runs compared were always parallel, it was not thought necessary to maintain a fixed temperature. The temperature, however, did not vary over  $2^\circ\text{C}$ . throughout the entire work; and not more than a few tenths of a degree during a given set of oxidations.

The samples of sugars used were obtained as follows: sucrose from the U. S. Bureau of Standards; fructose, from Merck; and glucose and mannose, from Kahlbaum. Fresh solutions of the glucose were made up from time to time.

### 4. *Rates of Oxidation of Glucose, Mannose, Fructose, and Sucrose.*

The chemical equation expressing the oxidation of glucose or mannose by iodine is as follows:



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<sup>7</sup> Hewitt, J. A., *Brit. Med. J.*, 1923, i, 590.

The reaction rate at any moment should therefore be proportional to the prevailing concentrations both of the sugar and of the iodine, provided the temperature remains constant, and the hydrogen ion concentration, which has a large effect on the rate, also remains unchanged. That is, representing by  $A$  and  $B$  the initial concentrations of the sugar and iodine, respectively, by  $x$  the fraction of the sugar transformed at any time  $t$ , by  $\frac{dx}{dt}$  the rate at which this fraction increases with the time, and by  $k$  the specific reaction rate at the given temperature and hydrogen ion concentration, the rate should be expressed by the equation:

$$A \frac{dx}{dt} = k (A - Ax) (B - Ax), \text{ or } \frac{dx}{dt} = k (1 - x) \left( \frac{B}{A} - x \right)$$

This equation yields on integration the following expression for the specific reaction rate.

$$k = \frac{0.4343}{t (B - A)} \log_{10} \frac{\left( \frac{B}{A} \right) - x}{\left( \frac{B}{A} \right) (1 - x)}$$

Reaction mixtures were made up in the case of each of these sugars by mixing the following solutions: 10.0 cc. of 0.1 per cent sugar solution; 10.0 cc. of 0.025 molal  $I_2$  in 0.06 molal KI; 15.0 cc. of 1 molal  $NaHCO_3$  or of 0.5 molal  $Na_2HPO_4$ ; and 15.0 cc. of water. The mixtures were, therefore, initially 0.00111 molal in monosaccharide, 0.00500 molal in  $I_2$ , and either 0.3 molal in  $NaHCO_3$  or 0.15 molal in  $Na_2HPO_4$ . Seven such mixtures of each sugar were placed in 100 cc. rubber stoppered conical flasks, and kept at the room temperature of 22–23°. From time to time one of the mixtures was removed, and 5 cc. of 6 N  $H_2SO_4$  were added, whereby the reaction was stopped; the decrease in the free iodine content was then determined by titrating it with a standard  $Na_2S_2O_3$  solution, which was 0.0200 molal in the  $NaHCO_3$  experiments and 0.0189 molal in the  $Na_2HPO_4$  experiments.

The results with glucose and mannose are given in Table I. In the columns headed  $100x$  are given the percentage of glucose oxidized as calculated from the decrease in iodine content; and in those headed  $k$ , the values of the specific reaction rate calculated by the above equation. The values given for the oxidation in the

$\text{NaHCO}_3$  solutions are the average of the three similar runs; those in the  $\text{Na}_2\text{HPO}_4$  solutions represent one set of determinations.

It will be seen from Table I that, as would be expected for a bimolecular reaction taking place under constant catalytic conditions and temperature, the values of the specific reaction rate  $k$  are fairly constant for both glucose and mannose in the presence of the hydrocarbonate. In the presence of the hydrophosphate, however, the values of  $k$  show a progressive decrease.

Similar runs with fructose and sucrose showed an iodine consumption at the end of 160 minutes equivalent to only 6 per cent oxidation of the sugar.

TABLE I  
*Rate of Oxidation of Glucose and Mannose by Iodine at 22-23°.*

Time	Glucose with $\text{NaHCO}_3$		Glucose with $\text{Na}_2\text{HPO}_4$		Mannose with $\text{NaHCO}_3$		Mannose with $\text{Na}_2\text{HPO}_4$	
	100 x	k	100 x	k	100 x	k	100 x	k
<i>m n</i>								
5	29 5	14 6	44 8	19 2	12 3	5 28	19 4	8 8
10	50 0	14 7	56 1	17 7	22 5	5 24	27 9	6 7
15	65 0	15 7	63 9	14 5	31 5	5 23	31 2	5 1
20	74 2	15 0	70 6	13 7	38 5	5 13	38 9	5 1
40	92 1	13 1	84 6	10 7	59 0	4 85	52 1	3 9
80	98 7		93 5		77 8	4 23	68 0	3 1
160	100 5		100 4		92 5		81 7	

These results conform with those of earlier investigators in that the aldoses are oxidized, while the ketose and biose remain practically unchanged. The small effect which is noted in the fructose and sucrose oxidations may well be due to experimental error. It is evident that even mannose and glucose differ greatly from each other in their rates of oxidation in both the hydrocarbonate and hydrophosphate solutions. The difference between the rates of glucose, mannose, and fructose should therefore be marked enough to indicate any transformation of glucose into isomeric hexoses which might occur in the presence of insulin.

#### *5. Oxidation of Glucose in the Presence of Insulin.*

The insulin used throughout the work was prepared by the process described by Collip<sup>3</sup> and was further purified by precipitation

<sup>3</sup> Collip, J B, *Tr. Roy. Soc. Canada*, 1922, xvi, series 3, section v.



from aqueous solution by changing the hydrogen ion concentration. The precipitate was washed with ether and dried over calcium chloride. A 0.1 per cent solution of the dried substance was made up by dissolving it in approximately 0.001 N hydrochloric acid. Previous tests showed that the insulin was stable in this solution. The activity of this insulin was tested on rabbits several times: 0.3 cc. per kilo weight of rabbit lowered the blood sugar of a normal rabbit from 110 to 45 mg. per 100 cc. in 1 hour, and the convulsive dose was between 0.3 and 0.4 cc. The insulin was also found to retain most of its activity on standing for 1 hour in solutions with an alkalinity similar to that of the oxidation mixtures. In all the calculations, the iodine taken up by the insulin

TABLE II

*Oxidation of Glucose by Iodine in Presence of Insulin.*

Time	Solution 0.30 molal in $\text{NaHCO}_3$			Solution 0.15 molal in $\text{Na}_2\text{HPO}_4$		
	Without insulin	With insulin	Difference	Without insulin	With insulin	Difference
<i>min</i>						
5	25.3	23.4	+1.9	34.0	34.9	-0.9
10	43.7	40.7	+3.0	49.3	47.8	+1.5
15	52.6	53.8	-1.2	57.9	57.2	+0.7
20	61.5	60.7	-0.8	64.9	64.2	+0.7
40	80.6	80.2	+0.4	79.5	79.7	-0.2
80	95.7	96.3	-0.6	92.2	91.6	+0.6
160	101.9	101.2	+0.7	96.2	96.8	-0.6

introduced into the reaction mixture was subtracted from the total iodine consumed.

The method of procedure was exactly the same as that described above. The reaction mixtures were made up just as in the experiments on the oxidation of the sugars alone; except that there were always run side by side duplicate mixtures differing only in the respect that one contained in the 50 cc. volume 1 cc. of 0.1 per cent insulin solution, and the other contained no insulin. The temperature was 22-23°.

Table II contains the results of these experiments. The numbers in the first column show the time elapsed after the mixing of the solutions; those in the other columns denote the percentage of glucose transformed.

It is evident from these results that insulin alone has no appreciable effect upon the oxidation of glucose by transforming it into either a more or less chemically reactive form. This is true even though a considerable excess of insulin was present over that required for the oxidation of the 10 mg. of glucose; thus the quantity was sufficient to lower the blood sugar of a 3 kilo rabbit 50 mg. per 100 cc. of blood in 1 hour.

*6. Oxidation of Glucose in the Presence of Liver Extract and Insulin.*

Both alcoholic and aqueous liver extracts were used in these experiments. The method of procedure was the same as that followed in previous experiments with the exception that the reaction mixtures were allowed to stand for 1 hour with the liver extract and insulin, before the addition of the iodine. A temperature of 22-24° was maintained by immersing the solutions in a water bath. As before, one-half of the solutions contained 1 cc. of 0.1 per cent insulin solution (in place of 1 cc. of water), in addition to the other ingredients, which were as follows: 10.0 cc. of 0.1 per cent glucose solution, 15.0 cc. of a solution 1.0 molal in  $\text{NaHCO}_3$  or 0.5 molal in  $\text{Na}_2\text{HPO}_4$ , 2.0 cc. of alcoholic or aqueous liver extract, 12.0 cc. of distilled water, and 10.0 cc. of a solution 0.05 N in  $\text{I}_2$  and 0.06 N in KI.

The alcoholic liver extract was prepared in the following manner. Fresh beef liver was ground and intimately mixed with an equal volume of 95 per cent alcohol. The juice was pressed out of the mass, diluted to 4 volumes, and filtered. The filtered solution was used in the oxidations.

The aqueous liver extract was prepared as follows: Equal volumes of fresh beef liver and 0.9 per cent sodium chloride solution were ground together thoroughly with sand in a mortar. The juice was then pressed out, diluted to 4 volumes, and filtered.

Table III contains the results of the experiments in which the alcoholic extract was used; and Table IV those in which the aqueous extract was used. The figures in the first column show the length of time of the oxidations; those in the other columns show the cubic centimeters of 0.025 molal  $\text{I}_2$  consumed in the various reaction mixtures. The second pair of experiments in each table was made with half the quantity of the liver extract.

These experiments show that glucose is unaffected by insulin in the presence of alcoholic or aqueous liver extract.

TABLE III.

*Oxidation of Glucose by Iodine in Presence of Alcoholic Liver Extract and Insulin.*

Time	Solution 0.30 molal in $\text{NaHCO}_3$			Solution 0.15 molal in $\text{Na}_2\text{HPO}_4$		
	Without insulin	With insulin	Difference	Without insulin	With insulin.	Difference.
<i>min.</i>						
5	1 63	1 69	-0 06	1 50	1 50	0 00
10	2 37	2 38	-0 01	2 09	2 08	+0 01
15	2 86	2 83	+0 03	2 36	2 38	-0 02
20	3 48	3 44	+0 04	2 75	2 71	+0 04
40	4 23	4 14	+0 09	3 36	3 37	-0.01
80	5 34	5 29	+0 05	4 14	4 12	+0 02
160	6 23	6 17	+0 06	4 93	4 84	+0 09
5	1 75	1 81	-0 06	1 46	1 45	+0 01
10	2 46	2 45	+0 01	1 91	1 90	+0 01
15	3 01	2 97	+0 04	2 23	2 20	+0 03
20	3 26	3 23	+0 03	2 43	2 42	+0 01
40	4 34	4 33	+0 01	2 97	2 94	+0 03
80	5 21	5 21	0 00	3 71	3 69	+0 02
160	5 41	5 41	0 00	4 46	4 39	+0 07

TABLE IV

*Oxidation of Glucose by Iodine in Presence of Aqueous Liver Extract and Insulin*

Time	Solution 0.30 molal in $\text{NaHCO}_3$			Solution 0.15 molal in $\text{Na}_2\text{HPO}_4$		
	Without insulin	With insulin	Difference	Without insulin	With insulin	Difference
<i>min</i>						
5	3 96	3 93	+0 03	3 51	3 49	+0 02
10	4 98	4 98	0 00	4 10	4 09	+0 01
15	5 35			4 40	4 38	+0 02
20	5 67	5 66	+0 01	4 74	4 72	+0 02
40	6 44	6 43	+0 01	5 46	5 27	+0 19
80	7 07	7 09	-0 02	6 25	6 24	+0 01
160	7 39	7 45	-0 06	7 02	7 01	+0 01
5	2 26	2 25	+0 01	1 52	1 49	+0 03
10	2 71	2 72	-0 01	1 93	1 90	+0 03
15	3 10	3 10	0 00	2 26	2 23	+0 03
20	3 47	3 48	-0 01	2 55	2 53	+0 02
40	4 00	4 02	-0 02	2 89	2 88	+0 01
80	4 61	4 64	-0 03	3 24	3 24	0 00
160	4 88	4 92	-0 04	3 59	3 60	-0 01

### 7. Oxidation of Glucose in the Presence of Insulin and Blood.

In order to make the work more complete, the effect of insulin on the oxidation of glucose in the presence of the blood serum and oxalated blood was studied. As in the other experiments, the results consistently indicated no change in the glucose on the addition of insulin.

The method of procedure was exactly the same as that followed in the work with liver extract. The reaction mixtures were changed only by substituting blood or blood serum for the liver extract.

Table V contains the results of the set of experiments in which 0.2 cc. of oxalated blood was added to each of the reaction mixtures. The temperature was 23.2–23.4°

TABLE V  
*Oxidation of Glucose in Presence of Oxalated Blood and Insulin*

Time	Solution 0.30 molar in $\text{NaHCO}_3$			Solution 0.15 molar in $\text{Na}_2\text{HPO}_4$		
	Without insulin	With insulin	Difference	Without insulin	With insulin	Difference
5	2.92	2.91	+0.01	2.51	2.48	+0.03
10	3.48	3.48	0.00	2.94	2.92	+0.02
15	3.83	3.89	-0.06	3.24	3.22	+0.02
20	4.07	4.12	-0.05	3.44	3.42	+0.02
40	4.75	4.77	-0.02	4.01	4.00	+0.01
80	5.20	5.23	-0.03	4.60	4.57	+0.03
160	5.64	5.64	0.00	5.03	5.05	-0.02

### SUMMARY.

It was first shown by this investigation that, in confirmation of the results of others, the rate of oxidation of various sugars by iodine in solutions of  $\text{NaHCO}_3$  or  $\text{Na}_2\text{HPO}_4$  varies greatly with the nature of the sugar; thus mannose was oxidized only about one-third as fast as glucose, and fructose and sucrose were scarcely oxidized at all, under the conditions of the experiments. This indicated that a study of the relative rates of oxidation of glucose before and after treatment of it with insulin would furnish a sensitive means of determining whether any of the glucose had been transformed by it into any other substance, even into a stereomeric hexose.

Strictly comparable experiments were therefore made with glucose alone, with mixtures of it with insulin, with insulin and liver extract, or with insulin and blood serum or oxalated blood. In no case was any difference detected in the rate at which the iodine is consumed. This shows that no appreciable reaction takes place between glucose and insulin even in the presence of the animal fluids mentioned. It indicates, therefore, that the metabolic process must be more complicated in character; also that there is little promise of developing a method of assay for insulin on the basis of its action on glucose in glass.

## ACID-BASE METABOLISM.

### I. DETERMINATION OF BASE BALANCE.\*

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Total acid-base metabolism in two infants has been calculated by chemical analysis of all acid and basic radicals in the food, urine, and feces. Changes in the acid-base content of the body and the mechanism whereby the organism maintains the normal reaction of the blood and tissues can thus be determined. The essentials for such measurements are: (1) to include all sources of acid or base—food, urine, and feces, (2) to quantitate all types of material which produce acids and bases—actual or potential; and (3) to state the results in comparable terms

1. *Sources of Acid and Base.*—That the data may have value, every significant source of acids or bases must be included. A sample of the actual food must be analyzed, samples vary so much that accepted standard analyses cannot be used. Analysis of the mineral content of the feces is necessary to determine the total acid-base metabolism. The urine has received most attention as a factor in excretion of acids and salts. Other sources of output might be vomitus or saliva. In our experiments, none occurred. The value of sweat and lacrimation was thought to be so small as to fall within the experimental error.

2. *Quantitative Analysis.*—The analyses cover the four basic elements: sodium, potassium, calcium, and magnesium; and the acid radicals: sulfates, phosphates, and chlorides. Sulfur and phosphorus must also be included to the extent to which they are oxidized in the body to form sulfate and phosphate—actual from

\* Read before the American Association of Biochemists, December, 1921, at the New Haven meeting.

potential acids. All these analyses can be made with a high degree of accuracy. Iron and other elements were not analyzed because they are present in minute amounts. Organic acids and organic bases are not taken into account. If ingested or formed in the body, they may be utilized or oxidized. If excreted, their effect is shown when the minerals are analyzed. Carbonates and ammonia are omitted for the same reason. Ammonia excretion is represented by an equivalent mineral acid excretion, and carbonates by a corresponding alkali excretion.

3. *Terms and Calculation* —Acidity does not refer, in this study, to the true acidity as measured by the hydrogen ion concentration. Milk is alkaline in respect to its minerals, but acid in reaction. Acid or base value means the difference between mineral acid radicals (except carbonates) and basic radicals (except ammonia) expressed in cc of 0.1 N. A plus sign indicates acid, a minus sign indicates base. Sodium, potassium, and chlorine are monovalent; calcium, magnesium, and sulfate are divalent; phosphate is calculated with a valence of 1.8—the extent to which it is neutralized in the blood.

The balance is positive when acid or base is retained, negative when they are excreted in excess of intake, when the body neither gains nor loses, it is in acid-base equilibrium. The value of acid-base metabolism is always stated in terms of base balance. Four possibilities thus occur.

1	Base retention	=	Positive base balance
2	Acid excretion in excess of intake	=	“ “ “
3	Base “ “ “ “ “	=	Negative “ “
4	Acid retention	=	“ “ “

### *Plan of Experiment.*

The investigation was carried out in the Department of Pediatrics, the Johns Hopkins University and the wards of the Harriet Lane Home, the Johns Hopkins Hospital.

Normal acid-base metabolism was studied for 3 day periods in three male infants aged 7, 9, and 7 months, who weighed 8.8, 5.5, and 5.7 kilos. Food, urine, and feces were analyzed and the acid-base value was calculated. Then, without alteration of other conditions, in one case acid was added, and in the other two alkali, and the metabolism was again determined.

1. *Material*.—As shown in the protocols, the babies had previously recovered from acute infections. The second baby was also underweight. There is no reason to believe that during the period studied their metabolism was abnormal. For a study of normals, babies of known history who gain at a normal rate are essential. To avoid daily fluctuations and to permit accurate collections of excreta—especially the feces—the metabolism periods should be as long as possible. Conditions should be uniform to permit the babies to attain equilibrium. The infants were placed upon the experimental diet 2 days before collection of material was begun. The diet was continued after the period until the last specimen of feces was obtained.

2. *Food*.—The food consisted of whole milk and water mixtures with added cane-sugar—considered suitable for the babies by Dr. John Howland. The food was made up in bulk, sufficient for the whole period of the experiment. The milk was sterilized daily for 3 days and kept on ice. A sample was taken for complete analysis. All food was well taken and no feedings were vomited or regurgitated. Acid or alkali was divided into equal portions for each feeding and was added to the milk immediately before it was fed. At the first and last feedings of the experimental period, ash-free carbon was added to mark the stools. The feeding was continued until the marked stool was obtained.

3. *Method of Collection*.—During the period of study the babies were on a metabolism bed, similar in principle to that described by Courtney and Fales (5), but slightly modified by Dr. J. L. Gamble. Every effort was made by the nurses to insure not only complete collection of specimens, but also for the best of nursing care. It is a pleasure to acknowledge our indebtedness to Miss Hamer, Miss Victor, and Miss Elliot for their excellent cooperation. We felt it was important for the baby to be comfortable and not constantly crying and fretting. The mattresses were so devised as to prevent exposure of the baby. The penis and not the whole scrotum was placed in an adapter attached to the collecting bottle. The buttocks were held in place over a small porcelain container and the stools, which were soft and formed, were received in it, without soiling the bed.

There was no known error in collecting the specimens. The urine was received into bottles containing 5 cc. of thymol chloroform and shaken every hour to insure contact with the preservative. The bottles were changed every 6 hours and put on ice. At the end of 24 hours the volume was measured, the bottles were rinsed with distilled water, and the urine was made up to 1,000 cc. The stools were weighed, put on ice as soon as passed, and after the end of the experimental period the baby was continued on the metabolism bed until the marked stool was obtained. The stool for the whole period was then mixed by putting it through a fine sieve and made up to 1,000 cc. The container was shaken for a half hour before taking a sample. This gave a thin emulsion which could be readily pipetted and analyses showed that this method gave closely agreeing duplicates. On both the urine and stools, the ammonia, acid titration, and fatty acids were done immediately so as to preclude the gross changes due to decomposition. The ash analyses were done at leisure.



4. *Methods of Analysis.*—In the urine, Na and K were determined as chlorides and then as the chloroplatinates, Na being calculated by difference (9). Ca and Mg were determined by McCrudden's method (18, 19). The Ca was ignited and weighed in platinum as CaO. The Mg was precipitated as  $\text{MgNH}_4\text{PO}_4$ , and weighed as  $\text{Mg}_2\text{P}_2\text{O}_7$ . The Cl was determined by McLean and Van Slyke's method (20), the  $\text{NH}_3$  by Folin and Macallum's method (11) with aeration and nesslerization, the total N by the Folin-Farmer (10) or by the Kjeldahl method, creatinine by Folin's method (8), the total S by Benedict-Denis' method (6); and the sulfate and ethereal sulfate by Folin's method (7). The total phosphorus was ashed by Neumann's method (21) and precipitated as molybdate and then as  $\text{MgNH}_4\text{PO}_4$ , and weighed as  $\text{Mg}_2\text{P}_2\text{O}_7$  (16). The  $\text{PO}_4$  was determined by the uranium acetate method (9), the  $\text{CO}_2$  by Van Slyke's method (31), organic acid by Black's method (1) of setting in plaster and extracting with ether, and ash by Stolte's method (29). The pH was determined by the colorimetric method of Clark (4), total acid excretion by the method of Henderson and Adler (15).

In the food and feces Na, K, Ca, and Mg in the ash were determined as above,  $\text{NH}_3$  as above, Cl as above after ashing with carbonate, total sulfur by Folin's peroxide method (7) and in the bomb by Sherman's method (24), total P as above, inorganic P by the micro method of Marriott and Haessler (17),  $\text{CO}_2$  as above, fatty acid by Saxon's method (23) and Black's method (1), and titration value by the method of Shohl and Marriott (28). pH studies by the electrometric method were begun but not completed on account of the war.

All the usual precautions of analytical procedure were used. All analyses were done in duplicate or triplicate. The weights were calibrated against Bureau of Standards weights, the volumetric ware was calibrated, all solutions were standardized, all methods checked against known amounts of pure substances, and blanks run on the materials.

5. *Discussion of Methods and Errors.*—The value of the deductions depends largely upon the sum of the errors of all methods. Calcium is an important example, for in the food it is 517/1,100, and in the stool it is 366/477 of the base. A 25 cc sample of milk was taken, the probable error of the determination was 0.3 mg in 43.4 mg, or 0.6 per cent. The total value, 517 cc, is therefore accurate,  $\pm 3$  cc of 0.1 N. The errors of the other methods have been evaluated in the same way. The square root of the sum of the squares gives the probable error as 8.5 cc of 0.1 N for the food; 8 cc. of 0.1 N for the urine, and 7 cc. of 0.1 N for the feces. The probable error of the base balance is then 13 cc. of 0.1 N—any value greater than that is outside the experimental error.

To the errors of the analyses must be added those of sampling, of collecting specimens, errors inherent in metabolism methods, loss by perspiration, error of feeding, etc. In all probability the largest error is due to individual variation. Such factors can be reckoned only when enough statistics are available. The total error can at present be but roughly approximated.

*Results.*

Of the eight metabolism periods studied, four are here discussed. The other four were rejected because the data were not so complete nor accurate and because the sample of milk used was not analyzed. All data are given in Tables VIII and IX.

The data for this study comprise: Period I, Baby A, age 7 months, weight 8.8 kilos, on a normal diet; Period II, Baby A on the same diet, plus 250 cc. of 0.1 N HCl; Period III, Baby B, age 9 months, weight 5.5 kilos, on a normal diet; Period IV, Baby B on the same diet, plus the equivalent of 473 cc. of 0.1 N  $\text{NaHCO}_3$ .

*Analysis of Food.*—That milk is an alkaline food is generally known from determinations by Sherman and Sinclair (26), and Sherman and Gettler (25). They analyzed the separate acids and bases and calculated their values in terms of normal solutions. They conclude that cow's milk has an alkali value of 237 cc. of 0.1 N per 1,000 gm. When this is recalculated, with the valence of 1.8 for phosphorus, the value is 422 cc. of 0.1 N per liter. Forbes (12) also calculated the value of milk, using König's analyses, and found a value of 203.1 cc. of 0.1 N alkali per 1,000 gm. of dry substance. Calculated as whole milk, this equals 425 cc. of 0.1 N base per 1,000 gm. of milk.

Table I shows the ash values of the several samples of milk used in our experiments, the values of König, referred to above, Sherman's figures, and the latest figures from Forbes and co-workers (13). Forbes' are included because they are probably the best extant and also to show how milk analyzed by the same technique in the same laboratory may vary and at times be acid.

The calculation of the ash value of milk raises several questions. Both Sherman and Forbes calculate the valence of phosphate at 2. At the pH of the blood, 7.4, the valence is 1.8, which value has been used in our calculations. In the second place, neither phosphorus nor sulfur is completely oxidized in the animal body, so it is not correct to assume that they form entirely  $\text{H}_2\text{SO}_4$  and  $\text{H}_3\text{PO}_4$ . The exact value to be assigned to them is difficult to ascertain, certainly subject to revision. We have assumed, lacking proper data, that they are burned in the stool in the same proportion as in the urine; the error cannot be large and an approximation is neces-

TABLE I—*Acid-Base*

Amount	König		Sherman		Forbes I		Forbes II		Forbes II	
	Per 1,000 gm		Per 1,000 gm		Per 1,000 gm		Per 1,000 gm		Per 1,000	
	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm.	cc 0.1 N	gm.	
Ash										
N.										
Ca	1 15	575	1 24	620	1 009	504	1 321	660	0 873	
Mg	0 112	92	0 11	92	0 131	109	0 163	136	0 150	
Na	0 436	190	0 69	300	0 376	180	1 094	475	0 334	
K	1 47	377	1 54	395	1 410	362	0 984	252	1 169	
Base		-1,234		-1,407		-1,155		-1,523		-
P	0 825	480	0 92	535	0 858	498	1 035	603	0 803	
PO <sub>4</sub>										
S	0 072	45	0 31	194	0 266	163	0 471	295	0 419	
SO <sub>4</sub>										
Cl	1 00	284	0 91	256	0 954	268	1 748	491	1 669	
Acid		+809		+985		+929		+1,389		-
Balance		-425		-422		-226		-134		

\* The values are calculated with the valence of phosphorus at 1.8. In determining the value for metabolism periods, the fraction phosphorus burned to phosphates was estimated at 90 per cent and the fraction of sulfur burned at 85 per cent.

† Values not used in calculation of acid value.

sary. Sherman and Gettler have correctly subtracted the non-sulfate sulfur in the urine in calculating the acid value in their metabolism experiments, but do not subtract it in the value assigned to the milk. For our purposes, we have used the proportion of inorganic sulfate as determined in our metabolism periods—90 per cent for phosphorus and 85 per cent for sulfur.

The present analyses agree satisfactorily with those cited. The calcium and phosphorus are each slightly high in one instance. The magnesium, sodium, and potassium are quite average. The sulfur seems high, but is within normal limits. Our figures show that in each 1,000 gm. of milk there are 1,325 cc. of alkali and 1,100 cc. of acid, as burned by the body. Of the alkali, the calcium is 55 per cent; and of the acid, the phosphate is 45 per cent. The value per 1,000 gm. of milk is 233 and 202 cc. of 0.1 N alkali.

*Cow's Milk.*

Period I	Period III		Period I *		Period II *		Period III *		Period IV *	
Per 1,000 gm	Per 1,000 gm		833 cc		833 cc		567 cc		567 cc.	
cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N
			6 202		6 902		4 620		6 760	
			4 27				2 73			
	620	1 34	625	517			0 775	388		
4	128	0 123	103	103			0 072	60		
3	220	0 530	230	183			0 310	135		473
	366	1 36	348	304			0 785	201		
	-1,334		-1,306	-1,107				-784		
	565	0 871	506	468†			0 505	293†		
				418				269		
	301	0 478	299	230†			0 279	174†		
				212				150		
	266	0 950	265	222	1 650	250	0 545	154		
	+1,132		+1,073	+852				+573		
	-202		-233	-255		-5		-211		-684

*Metabolism of Acids and Bases.*

*Total Intake*—The diet consisting only of milk and cane-sugar was alkaline; the amount of alkali depended upon the quantity of milk used. In the first period, 833 cc of milk were given, with a value of 255 cc of 0.1 N base, in the second, the same diet plus 250 cc of 0.1 N HCl = 5 cc of 0.1 N base, in the third, 577 cc. of milk, with a value of 211 cc of 0.1 N base, in the fourth, the same diet plus 473 cc of 0.1 N NaHCO<sub>3</sub> = 684 cc. of 0.1 N base.

*Excretion in the Urine*—Heretofore studies on acid excretion have been limited largely to the urine. For a correct interpretation, the stools must also be taken into account, and each must be regarded separately in relation to the intake. The balance for food and urine is given in Table II. The urine has an acid value. Acid increases the acid output; when acid is added to the intake, all is excreted. When alkali is given, the urine becomes alkaline and base is retained. A large positive base balance results in all cases.

*Excretion in the Feces.*—The positive base balance for food and urine alone (300 to 500 cc. of 0.1 N) is so large as to indicate that excretion of minerals by the feces must be of considerable importance. The word excretion is used to include everything in the feces—whether excreted, secreted, or unabsorbed. The acid or alkali value of the stool is calculated in the same way as for the

TABLE II.

*Balance of Urine and Food in Cc. of 0.1 N.*

Period ..	I Normal	II 250 cc HCl added	III Normal	IV 473 cc NaHCO <sub>3</sub> added.
	cc 0.1 N	cc 0.1 N	cc 0.1 N	cc 0.1 N
Bases (1)	-357	-440	-289	-649
Urine acids (2) . . . . .	+570	+909	+366	-376
Value of urine (3) = (1) - (2)	+213	+469	+77	-273
" " food (4)	-255	-5	-211	-654
Positive base balance for food and urine (5) = (3) - (4) ..	468	474	288	381

TABLE III.

*Balance of Feces and Food in Cc. of 0.1 N.*

Period . . . . .	I Normal	II 250 cc HCl added	III Normal	IV 473 cc NaHCO <sub>3</sub> added
	cc 0.1 N	cc 0.1 N	cc 0.1 N	cc 0.1 N
Bases (1) . . . . .	-477	-560	-332	-405
Feces acids (2) . . . . .	+123	+168	+90	+120
Value of feces (3) = (1) - (2) . . . . .	-354	-392	-242	-285
" " food (4) ..	-255	-5	-211	-654
Base balance for food and feces (5) = (3) - (4) .. . . . . .				

Neg 99 Neg 387 Neg 31 Pos 369

urine and compared with the value of the food. The data are shown in Table III.

The feces have an alkali value and acid does not increase the acid excretion. Alkali increases alkali excretion by the bowel. A large negative base balance results in every case but the alkali period; when alkali is added, 400 cc. are retained in excess of nor-

mal and the balance becomes positive. The action of the bowel is, in the main, just the opposite of that of the kidney. The kidney normally excretes the acid elements in excess. The bowel excretes the alkali in excess. Added acid is excreted almost wholly by the kidney. Added alkali is excreted partly by the kidney and partly by the bowel.

The bowel acts in maintaining neutrality of the body by removing alkalis. When excessive alkali is given, the amount of the output is increased. Whether the bowel can also serve as an emergency factor in excreting excess acid remains yet to be proved. For in the case given above, even after acid was added, the food was still neutral ( $-5$  cc. of  $0.1$  N). Analyses of the stool in acidosis give reason to believe that the bowel can also act in excreting acid.

TABLE IV  
*Base Balance per Day.*

Period	I Normal	II 250 cc HCl added	III Normal	IV 473 cc NaHCO <sub>3</sub> added.
	cc 0.1 N	cc 0.1 N	cc 0.1 N	cc 0.1 N
Value of urine (1)	+213	+469	+77	-273
" " feces (2)	-354	-392	-242	-285
Value of output (3) = (1) - (2)	-141	+77	-165	-558
" " food (4)	-255	-5	-211	-684
Positive base balance (5) = (3) - (4)	114	82	46	126

It has been claimed that the main changes taking place in the bowel are due to bacterial action and, therefore, do not represent any adequate picture of what the body has absorbed or excreted. Such reasoning forced us to the present method of study which eliminates organic acids formed by bacteria. Bacteria do not make sodium or sulfur; but they may make sulfate into sulfur, or sulfur into sulfate. Fortunately for the outcome of the work, the proportion due to sulfur and phosphorus in the stool is so small in comparison to the bases that this becomes quantitatively a minor problem. Future work may modify the picture, but cannot greatly change it.

*Base Balance.*—The data necessary for determining base balance are given in Table IV. The combined acid excretion of the urine and feces is compared with the value of the food.

We can draw the conclusions: (1) A positive base balance results in all cases. (2) Acid decreases the positive base balance. When 250 cc. are given, the balance is diminished one-third; 32 cc. of acid are retained, one-seventh of the intake. (3) Alkali increases the positive base balance. When 473 cc. are given, the balance is increased twofold; 80 cc. of alkali are retained, one-sixth of the intake. (4) On the milk diet, the output is alkaline in value; but when acid is added to the milk, the output is acid.

Normally a retention of base must occur. The body fluids are alkaline; if their alkalinity is to be preserved, base must be stored in growth. Depletion of the alkali stores causes acidosis, increase, an alkalosis. As is to be expected, the figures show a smaller base retention when acid is given and a larger retention when alkali is given. The average base retention is approximately 75 cc. of 0.1 N base per baby per day.

TABLE V  
*Base Balance per Kilo per Day in Cc of 0.1 N.*

Period	I Normal	II 250 cc HCl added	III Normal	IV 473 cc NaHCO <sub>3</sub> added
	cc 0.1 N	cc 0.1 N	cc 0.1 N	cc 0.1 N
Value of food (2)	-28	-0 5	-38	-124
" " output (1)	-16	+9 0	-30	-101
Positive base balance (3) = (1) - (2)	12	9 5	8	23

A more accurate comparison is made if the retention is calculated in relation to body weight. Baby A weighed 9.0 kilos at the end of the experiment, and Baby B, 5.5 kilos. On the *per kilo basis*, the values are given in Table V. The positive base balance on a normal diet is in one case 12 cc., and in the other 8 cc. per kilo per day.

This method of computation can be applied to values given in the literature, if the milk, urine, and feces have been analyzed completely and by approved methods. Four studies are available. Blaumberg studied a baby on cow's milk (2). The baby was 7½ months old and weighed 7.15 kilos. He gained 21.9 gm. daily. Negative balances of sodium, sulfur, and chlorine occur, although the baby was gaining in weight. Losses of sulfur and chlorine in ashing the milk probably account for these negative balances.

If Forbes' or our values for the milk be substituted, the balances become positive. The calcium, too, seems slightly high and the magnesium slightly low. To the value obtained, the values of the negative retention have been added on the assumption that the intake figures are too low, at least by that amount. The results show a retention of 26 cc. of 0.1 N alkali per kilo per day. If a normal retention of phosphorus and sulfur occurred, the retention would be less alkaline, and would approximate that of the following cases.

The breast-fed baby studied by Blauberg (3) was  $4\frac{3}{4}$  months old, weighed 6.7 kilos, and gained 10 gm. daily. The results are more uniform and the retention calculated in acid-base value is 52 cc. of 0.1 N base, or 8 cc. of 0.1 N alkali per kilo per day.

The breast-fed baby of Tobler and Noll (30) was  $2\frac{1}{2}$  months old, weighed 4.0 kilos, and gained 25 gm. daily. The metabolism resulted in a positive base balance of 75 cc., or 18.2 cc. of 0.1 N per kilo. This is larger than for the baby studied by Blauberg; but the gain in weight was proportionally greater. Also, even though the baby was smaller, he took more food. Therefore, it is probable that the difference in the base balance of the two babies represents a corresponding difference in their metabolism.

Sawyer, Baumann, and Stevens (22) have made a metabolism study on two boys aged 5 and 8 years, weighing 22.7 and 23.0 kilos. We have recalculated their figures, not considering  $\text{NH}_3$  as an alkali, and have computed the acid-base value of the food which they analyzed. The figures for the normal periods resolve into a positive base balance of 10.0 and 14.5 cc. of 0.1 N alkali per kilo per day. Of course, the children are older; however, the base balance is nearly the same as for infants.

In our study the base balance was reduced from 12.0 to 9.5 cc. of 0.1 N when 250 cc. of HCl were given. The base balance was increased from 8 to 23 cc. of 0.1 N when 473 cc. of  $\text{NaHCO}_3$  were given. No data are available with which the acid and alkali periods can be compared. There is, however, the study of Sawyer, Baumann, and Stevens. They have, in the same two children studied during normal periods, determined the effect on mineral metabolism of acidosis produced by high fat feeding. Clinical symptoms of acidosis were produced. The alkali reserve, nor-



mally 50 and 49 volumes per cent, was reduced to 29 and 37 volumes per cent. Calculation of the mineral metabolism shows that in the first case the positive base balance of 10.0 cc. of 0.1 N was changed to a negative base balance of 0.8 cc. of 0.1 N; in the second, the positive base balance was reduced from 14.5 to 6.0 cc. of 0.1 N. The increase or decrease in the base balance shows the development of alkalosis and acidosis.

To calculate the base retention compared to the gain in weight would be desirable. The actual gain, however,—50, 33, 10, and 20 gm. per day—seems too small in comparison to the errors in weighing and the errors caused by a full bladder or rectum, so that accurate calculation cannot be made. The base balance calculated in relation to ash retention—the number of cc. of 0.1 N base retained per gm. of ash retained—is shown in Table VI.

TABLE VI  
*Ash Balance.*

Period	I Normal	II 250 cc HCl added	III Normal	IV 473 cc NaHCO <sub>3</sub> added
Positive ash balance, gm	0.753	0.515	0.565	0.291
“ base balance per gm of ash balance, cc	150	158	82	430

For each gram of ash retained in a normal period, approximately 100 cc. of 0.1 N base are retained. Acid does not change greatly the character of the salt retained, but alkali makes the retention five times as alkaline as normal. The nitrogen metabolism studied in conjunction with the mineral balance is an aid in interpretation. A positive nitrogen balance indicates the extent of protein storage. If we assume that 1 per cent of body protein is sulfur, then daily 0.03 gm. of non-sulfate sulfur is stored. This sulfur has acid value only when it is burned during protein catabolism. Phosphorus must be similarly stored in non-acid form for nucleotides and phosphatides. Hence in calculating the value of sulfur and phosphorus for retention, the neutral portions must be determined and deducted.

*Neutral Salt Effect.*—The salts in cow's milk total more than twice those of breast milk. This has been given as one of the reasons for diluting milk in infant feeding. The actual retention of salts is nearly the same, but the percentage of retention is much greater for breast milk; therefore, the German pediatricians have tacitly assumed that the salts of milk form a *luxus consumption* and are utilized in respect to their needs. A positive base balance does not imply that no acid radicals are retained, but only that basic radicals are retained in excess. A positive balance results if either alkali is retained or acid excreted: but as salt

TABLE VII .

Period	Alkali				Acids			
	I	II	III	IV	I	II	III	IV
	Normal.	250 cc HCl added.	Normal	473 cc NaHCO <sub>3</sub> added.	Normal	250 cc HCl added.	Normal.	473 cc NaHCO <sub>3</sub> added.
Value of food (1)	-1,107	-1,107	-784	-1,257	+852	+1,104	+573	+573
" " output (2)	-824	-1,000	-621	-1,054	+693	+1,077	+456	+496
Value of retention (3) = (1) - (2)	-283	-107	-163	-203	+159	+27	+117	+77

retention determines the relation of alkali to the neutral salts, or to the osmotic pressure, effect on the body is quite different. The simple statement of this effect is included in Table VII.

In the acid period both fewer acid radicals and fewer alkali radicals are retained than in the normal period (and the sum of the two results in a smaller positive base balance). More neutral salts are therefore excreted and the salt retention is less than normal. In the alkali period, however, more alkali radicals and fewer acid radicals are retained than normal. The alkali radicals replace the acid radicals in the body.

TABLE VIII  
Acid-Base Value of Urine.

Period. . . .	I Normal		II 230 cc 0.1 N HCl added		III Normal		IV 473 cc 0.1 N NaHCO <sub>3</sub> added		V Normal		VI Normal		VII 600 cc 0.1 N HCl added.		VIII 300 cc 0.1 N NaHCO <sub>3</sub> added	
	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N
Amount . . .	3 153	495	3 306	735	2 186	510	4 474	500	3 18	586	1 776	510	2 664	2 86	457	
Ash . . . . .		6 1		6 0		6 4		8 2		6 6		6 8		8 4	8 6	
pH . . . . .		100		137		38		41		58		43		180	91	
Titratable acid. . .		160		374		124		37		105		171		301	269	
NH <sub>3</sub> . . . . .	0 224		0 524		0 174		0 052		0 146		0 240		0 430	0 376		
N . . . . .	3 40		3 66		1 89		1 96		1 88		1 92		2 14	1 68		
NH <sub>3</sub> , per cent. . .	5 7		14 3		9 2		0 03		8 3		12 4					
N, per cent. . .										70		84		56		
Organic acid. . .		5		6		29		300								
CO <sub>2</sub> . . . . .	0 118		0 129		0 078		0 082		0 072		0 086		0 094	0 097		
Creatinine . . .																
Ca . . . . .	0 017	9	0 022	11	0 026	13	0 021	10	0 078	39	0 022	11	0 017	9	0 033	16
Mg . . . . .	0 018	15	0 018	15	0 023	19	0 005	4	0 016	14	0 012	10	0 015	13	0 014	12
Na . . . . .	0 300	131	0 475	206	0 308	134	1 137	494	0 585	253	0 182	79	1 52	661	0 731	318
K . . . . .	0 785	202	0 813	208	0 478	123	0 550	141	0 475	121	0 539	138	0 515	132	0 526	135
Bases (1) . . .	-357			-440		-289		-649		-427		-238		-815		-481
P . . . . .	0 457		0 544													
PO <sub>4</sub> . . . . .	0 441	256	0 522	320	0 270	157	0 260	150	0 371	216	0 347	202	0 333	195	0 316	184
S . . . . .	0 241		0 260		0 147		0 153									
Ethereal SO <sub>4</sub> . .			0 011		0 002		0 006									

Inorganic SO <sub>4</sub> . . .	0 208		0 214		0 126		0 132		0 096		0 098		0 096		0 080		50
Total SO <sub>4</sub> .....	0 206	130	0 225	134	0 128	79	0 138	86	60				61		59		
Cl..... . .	0 657	184	1 615	455	0 454	130	0 528	140	340	1 20	0 406	114	0 437	123	0 267		75
Acids (2).....		+570		+909		+366		+376	+616			+377		+377		+309	
Balances (3) =																	
(1) - (2).....		+213		+469		+77		-273	+189			+139		-438		-172	

TABLE IX.  
Acid-Base Value of Feces and Base Balance.

Period .....	I Normal		II 250 cc 0.1 N HCl added.		III Normal		IV 473 cc 0.1 N NaHCO <sub>3</sub> added		V Normal		VI Normal		VII 600 cc 0.1 N HCl added		VIII 300 cc 0.1 N NaHCO <sub>3</sub> added.	
	gm.	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N	gm	cc 0.1 N
Ash.....	2 296		3 081		1 513		2 015		0 896		1 556		2 086		1 899	
N.....	0 285		0 385		0 18		0 24									
Titratable alkali.....		32		93		70		67		80		100		166		126
Fatty acid.....		250		220		69		100								
Ca.....	0 734	366	0 865	434	0 525	263	0 702	350	0 652	326 0	675	338 0	693	347 1	0 222	514
Mg.....	0 060	50	0 081	67	0 052	43	0 014	11	0 030	23 0	0 43	36 0	0 55	46 0	0 40	33
Na.....	0 079	34	0 073	32	0 025	11	0 063	28	0 041	18 0	0 33	14 0	0 65	28 0	0 28	12
K.....	0 104	27	0 104	27	0 060	15	0 063	16	0 047	12 0	0 127	33 0	0 165	42 0	0 085	22
Base (1).....		-477		-560		-332		-405		-381		-421		-463		-581
P.....	0 210		0 140		0 143		0 180			100		100		100		100
PO <sub>4</sub> .....		109		73		75		94								
S.....					0 014		0 019									
SO <sub>4</sub> .....	10		10		10		12		10		10		10		10	10
Cl.....	0 014	4	0 302	85	0 018	5	0 049	14	0 032	90	0 38	11 0	0 40	11 0	0 53	15
Acid (2) .....		+123		+168		+90		+120		+119		+121		+121		+125
Feces balance (3) = - (1) - (2).....		-354		-392		-242		-285		-262		-300		-342		-456

Urine balance (4) (from Table VIII).....	+213	+469	+77	-273	+189	+139	-438	-172
Excretion (5) = (3) + (4)	-141	+77	-165	-558	-73	-161	-780	-628
Food value (6) (from Table I).....	-255	-5	-211	-684				
Positive base balance = (6) - (5).....	114	82	46	126				
Positive base balance per kilo.....	12.7	9.1	8.4	23.0				

## CONCLUSION.

Base balance defines a new physiological constant. Just as the nitrogen requirement can be definitely calculated, the acid-base requirements can be evaluated and the limits of normal defined. This is especially important in early life when normal growth must require its quota of alkali. Estimation of the base balance is significant in such diseases of metabolism as acidosis, rickets, and tetany, and should provide us with a new view-point as to their pathological physiology.

## SUMMARY.

1. A method of measuring base balance is outlined.
2. In babies under 1 year the normal positive base balance is 10 cc.  $\pm$  2 cc. of 0.1 N per kilo per day.
3. Acid or alkali when added also affect the character of the salt retention. Acid causes a smaller salt retention than normal, and alkali causes a salt retention approximately normal in amount, but more alkaline in substance.
4. Acid or alkali when added are retained to about one-seventh or one-eighth. The excretion of added acid is by way of the kidney; of added alkali, partly by the kidney and partly by the bowel. 250 cc. of 0.1 N HCl lower the positive base balance from 12.0 to 9.5 cc. of 0.1 N per kilo; 473 cc. of 0.1 N  $\text{NaHCO}_3$  raise it from 8.0 to 23.0 cc. of 0.1 N per kilo.

It is a pleasure to acknowledge the assistance of Dr. John Howland and Dr. W. McKim Marriott under whose direction this work was conducted.

*Protocols.*<sup>1</sup>

*Baby A. Period I.* Baby received food specially prepared and analyzed in laboratory, beginning Jan. 17. Five feedings of 225 cc. each. The feeding consisted of whole milk, 833 cc., water, 292 cc., and cane-sugar, 56 gm., equivalent to 85 calories per kilo. Baby weighed 8.8 kilos. Put on metabolism bed at 1 p.m., Jan. 10. The carbon was put into the 6 p.m. feeding and the urine collected from that time. The stools were obtained as follows: 3 and 8 a.m., rejected; 2 p.m., part containing carbon retained; 4 and 8 p.m., Jan. 20; 4, 6, and 8 p.m., Jan. 21; 6 and 8 a.m. and

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<sup>1</sup> The clinical histories are omitted at the request of the editors

5.30 p m., Jan. 22, 12 and 2 p.m., Jan. 23. The 6 p.m. stool contained the carbon given at 6 p.m. the day before, when the urine sample was complete. The weight of the eleven moist stools was 95 gm. The urine was divided in 24 hour samples: 510, 480, 480, and 510 cc. Average, 495 cc. of urine per day. There were no known errors. Removed from frame 2 p.m., Jan. 23. Weight 8.95 kilos

*Baby A. Period II.*—Same baby as in Period I. Weight 8.9 kilos Jan. 24, 1917 put on frame at 2 p.m. Same feeding as in previous period. To this was added HCl. The feeding was divided in two portions and the equivalent of 50 cc. of 0.1 N acid in 15 cc. of fluid was added to the first portion and fed at once to the baby. The second portion was used to rinse the flask in which the acid had been and then given to the baby. The feeding was well taken. There were no signs of hyperpnea. At 6 p.m., Jan. 25, carbon was given and the collection of specimens begun. At 10 p.m. the acid was omitted by mistake, but given at 2 a.m. with half of the morning bottle. Jan. 26, 6 a.m., half the usual feeding, plus 50 cc. of 0.1 N HCl. The baby is pale and the ears are discharging freely. Jan. 27, ears better. Baby in good condition. Respirations 60. Carbon given at 6 p.m. Last marked stool obtained at 11.40 a.m., Jan. 28. At 2 p.m. baby vomited about an ounce of feeding. As the stool had been previously obtained, the experiment was stopped and that day's urine rejected. 8 p.m., temperature 103° F. Stools obtained at 6 p.m. (partly marked with carbon), Jan. 27, 1 a.m., 12 n., 2 and 6 p.m.; Jan. 28, 11.40 a.m., part marked with carbon. Total moist weight 72 gm. Urine Jan. 570, 712, and 760 cc. Weight 9.0 kilos.

*Baby B. Period III.*—The experiments were conducted exactly as in Periods I and II. Mar. 12, baby put on frame at 2 p.m. Weight 5.5 kilos. Feeding consisted of milk, 585 cc., water, 315 cc., cane-sugar, 45 gm. Five feedings of 180 cc. each. Baby off frame at 10 a.m. on Mar. 16. Carbon stool obtained. No known errors. Weight 5.53 kilos.

*Baby B. Period IV.* This period was begun at 6 p.m., Mar. 16. In this period 3.97 gm. of  $\text{NaHCO}_3$  in 50 cc. solution were given each day—2 cc. in each feeding. Weight at beginning 5.45 kilos. Mar. 20, carbon stool obtained. Weight 5.5 kilos.

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## ACID-BASE METABOLISM.

### II. MINERAL METABOLISM.

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An attempt to evaluate the total effect of acid and base on mineral metabolism of infants was presented in the first paper. The present study deals with the more minute effects—the balance and paths of excretion of the individual elements. The material and periods of study are the same.

Infants on a normal diet should show a positive balance of each element, which must be affected by the amount of intake, the metabolic requirements, and the relation to other elements. Data in terms of 0.1 N solutions are presented to show: (1) the intake of the individual elements; (2) the excretion of each in the urine and feces; and (3) the balance. All salts leaving by way of the bowel are called "excretion." In practically all the literature the amount of minerals in the feces is considered as not assimilated and the percentage retained is given as per cent of "absorbed." As in previous experiments, all our values for retention and excretion are calculated in relation to the amount ingested.

*Ash.*—The simplest, most accurate, and most comprehensive understanding of mineral metabolism is furnished by the ash values of food, urine, and feces. The determination is easy. The interpretation is difficult. Albu and Neuberg (1) have pointed out that the ash does not represent the elements in the food since much of the chlorine, sulfur, and sodium may be lost in ashing. This criticism does not apply for ashing done by the Stolte method. However, the ash contains mixtures of oxides and carbonates impossible to calculate. The phosphates are converted into pyro- and meta-phosphates in unknown proportions. Conditions for the formation of oxides, carbonates, and phosphates differ in the

food, urine, and feces, and the results of their ash analyses are not at all comparable. Deductions based upon the analysis of the ash should be guarded.

Our figures for the ash values are given in Table I. The ash value of the food in Period II (acid) was calculated by adding to the ash, as determined for Period I (normal), the weight of the HCl and subtracting an equivalent amount of O<sub>2</sub>. In Period IV (alkali) the weight of the bicarbonate, 4 gm., was calculated as carbonate, 2.529 gm., and added to the value of the ash in Period III (normal). Both acid and alkali seem to diminish the ash retention, both absolutely and as percentage of intake. Yet, first analyses showed that though added acid removes basic ions and alkali removes acid ions, alkali in excess of acids is retained in both cases. An actual increase of ash retention would be expected

TABLE I

*Ash*

Period	Intake	Feces		Urine		Total excretion		Retention	
	<i>gm</i>	<i>gm</i>	<i>per cent</i>	<i>gm</i>	<i>per cent</i>	<i>gm</i>	<i>per cent</i>	<i>gm</i>	<i>per cent</i>
I	6 202	2 296	37	3 153	51	5 449	88	0 753	12
II	6 902	3 081	44 5	3 306	48	6 387	92 5	0 515	7 5
III	4 260	1 513	35 5	2 186	51 5	3 699	87	0 561	13
IV	6 780	2 015	30	4 474	66	6 489	96	0 291	4

when alkalies are given. The effect on the paths of excretion shows that in a normal period about 37 per cent of the ash is excreted by stool and 51 per cent by urine. In the acid period, excretion by the stool is increased from 37 to 44 per cent and by the urine decreased from 51 to 48 per cent. In the alkali period, the reverse is true; excretion in the stool is decreased from 35 to 30 per cent of intake, and in the urine increased from 51 to 66 per cent.

*Nitrogen.*—The nitrogen results are, of course, much simpler to interpret than the total ash and in these values considerable confidence can be placed. The nitrogen intake (Table II) is high, as is to be expected from a milk diet. For an adult of 70 kilos with intake proportional to weight, this would represent 33 and 35 gm. of N, or 208 and 216 gm. of protein, respectively, for the normal period. The retention is approximately normal for

infants—0.5 to 0.6 gm. of N, about 4 gm. of protein per day. It is nearly the same whether the intake be high or low; if low a larger percentage is retained. Both acid and alkali diminish the retention. In the acid period it is diminished from 13.5 to 5.5 per cent of the intake and in the alkali period, from 24.5 to 9.5 per cent of the intake.

The excretion of N is largely through the urine. The percentage found in the stool is larger in infants than in adults. Both acid and alkali increase the excretion of nitrogen in the urine and

TABLE II

*Nitrogen*

Period	Intake	Feces		Urine		Total excretion		Retention	
		gm	per cent	gm	per cent	gm	per cent	gm	per cent
I	4 27	0 285	7	3 40	79 5	3 685	86 5	0 585	13 5
II	4 27	0 385	9	3 66	86	4 045	94 5	0 235	5 5
III	2 73	0 18	6 5	1 89	69	2 07	75 5	0 66	24 5
IV	2 73	0 24	9	1 96	71 5	2 20	80 5	0 53	9 5

TABLE III

*Calcium*

Period	Intake		Excretion						Retention		
			Feces			Urine					
	gm	cc	gm	cc	per cent	gm	cc	per cent	gm	cc	per cent
I	1 031	517	0 734	366	71	0 017	9	2	0 280	140	27
II	1 031	517	0 865	434	84	0 022	11	2	0 144	72	14
III	0 775	388	0 525	263	68	0 026	13	3	0 224	112	24
IV	0 775	388	0 702	350	90	0 021	10	3	0 052	26	7

also in the stool, for our experiments, in roughly the same proportion. In addition to the total nitrogen the effect on  $\text{NH}_4$  nitrogen and creatinine N is shown in Table VIII on page 248 of the first paper.

*Calcium.*—More interest has been shown in the calcium balance than in any other single element of mineral metabolism. The results of our determinations are shown in Table III. If we take the standard for a 70 kilo man as 1.50 gm., CaO equals 1.07 Ca, or 0.0152 gm. of Ca per kilo. Then it should be 0.137 and 0.0835

gm. of Ca for our babies, respectively. Since babies retain larger amounts of the intake to meet their greater needs for growth, this figure would be accepted as very low by most pediatricians. The intake for our babies was 1.031 and 0.775 gm., respectively. The actual need of calcium for infants, as determined by metabolism experiments, remains to be established.

The retention in the babies studied was decreased by acid from 140 to 72 cc. of 0.1 N and by alkali from 112 to 26 cc. of 0.1 N, but was still positive. Acid increased and alkali decreased the excretion of calcium in the urine. In infancy a much larger percentage leaves the body by the bowel than in adult life. In normal periods, 95 to 98 per cent of excretion were in the stool. Both acid and alkali increased the excretion of calcium in the stool, but a far greater amount, absolutely and relatively, than in the urine.

TABLE IV.  
*Magnesium*

Period	Intake		Excretion						Retention		
			Feces			Urine					
			gm	cc	per cent	gm	cc	per cent			
I	0 123	103	0 0599	50	48	0 0178	15	15	0 0457	38	37
II	0 123	103	0 0805	67	65	0 0179	15	15	0 0246	21	20
III	0 0720	60	0 0515	43	73	0 0226	19	32	0 0021	-2	-3
IV	0 0720	60	0 0135	11	19	0 0054	4	7	0 0521	45	74

*Magnesium.*—Magnesium metabolism is much less than the calcium, as shown in Table IV. The intake is roughly one-fifth that of calcium. Magnesium is also excreted largely through the feces—70 to 75 per cent in normal periods. Acid increases the total excretion and the percentage in the feces, but the urine is unaffected. Alkali decreases the excretion in both urine and feces to one-fourth of normal. The retention is diminished by acid to one-half of normal; alkali very largely increases it. Since the balance of magnesium is negative in the second normal period, this effect is perhaps partly compensatory and larger than if due to alkali alone. However, alkali causes a retention of 74 per cent of the intake, a large positive balance—45 cc.—as compared with 38 cc. during a normal period of the other baby with twice the magnesium intake. When the calcium retention is dimin-

ished by alkali, the magnesium is increased; acid diminishes the retention of both to about half the normal.

The ratios of excretion of the divalent elements show that magnesium exceeds calcium in the urine, as has previously been pointed out by Renvall, Bunge, and Von Noorden. The ratios of retention indicate that the magnesium is retained in excess of the amount necessary for bone; a greater proportion is then allowed for the soft parts.

*Sodium.*—The sodium results are shown in Table V. The intake would represent 8.3 to 6.29 gm. of NaCl, or 3.3 to 2.4 gm. of Na for a 70 kilo man, which would be considered small. Excretion is largely by way of the urine; in the normal babies, 79 to 92 per cent.

Acid markedly increases the excretion in the urine and the bowel shows a constant amount. In the acid period total excre-

TABLE V.  
*Sodium*

Period	Intake		Excretion						Retention		
			Feces			Urine					
	gm	cc	gm	cc	per cent	gm	cc	per cent	gm	cc	per cent
I	0 422	183	0 0785	34	19	0 300	131	71	0 0435	19	10
II	0 422	183	0 0732	32	18	0 475	206	112	0 126	55	30
III	0 310	135	0 0252	11	8	0 308	134	99	0 0232	10	7
IV	1 400	616	0 0629	28	45	1 137	494	81	0 200	89	14 5

tion increases by 50 per cent. In the alkali period, the picture is hardly comparable—the alkali used was sodium bicarbonate which increased the intake of sodium by more than 300 per cent. Under these conditions a smaller percentage of the intake was excreted both by the stool and urine. Of the 473 cc. of sodium added, 17 cc. left by the bowel and 360 cc. by the urine.

The retention is very sharply affected by acid and alkali. In Period II, 250 cc. of 0.1 N HCl cause a positive balance of 19 cc. to turn to a negative balance of 55 cc. The negative balance of 10 cc. is changed to a positive balance of 99 cc. by 473 cc. of 0.1 N NaHCO<sub>3</sub>. Thus, whether or not the effect is due to the particular acid or alkali used, the magnitude and direction of the result is unmistakable. The sodium balance is very markedly affected by additions of acid or alkali.



*Potassium*.—Potassium intake seems large, but is apparently not abnormally high for a baby. Calculated for a 70 kilo adult it equals 17.5 and 19.0 gm. of KCl, or 9.29 and 10.9 gm. of K per day.

The excretion of potassium in babies is largely by way of the urine, 88 to 88.5 per cent leaving the body by that path. Surprising as it may seem, neither additional acid nor alkali appreciably alters the excretion. The acid effect is negligible; alkali increases excretion in the urine 15 per cent. The figures accord with the theory of Bunge—that sodium drives out potassium. The experiment is a test of the hypothesis under severe conditions: on the one hand, sodium should cause the excretion of potassium; and on the other, potassium should be retained along with sodium in alkali feeding. Retention of potassium is the result of the two

TABLE VI

*Potassium*

Period	Intake		Excretion							Retention		
			Feces			Urine						
	gm	cc	gm	cc	per cent	gm	cc	per cent	gm	cc	per cent	
I	1 182	300	0 104	27	9	0 785	202	67	0 293	71	24	
II	1 182	300	0 104	27	9	0 813	208	69	0 265	65	22	
III	0 783	201	0 060	15	7 5	0 478	123	61	0 245	63	31 5	
IV	0 783	201	0 063	16	8	0 550	141	70	0 170	44	22	

causes. The tendency of alkali to cause potassium retention is less than the force which sodium exerts in causing the excretion of potassium.

The retention is also remarkably constant. The body retains 71 and 63 cc. of 0.1 N potassium in the normal periods, although this represents 24 and 31 per cent of the intake, respectively. The demand for potassium seems to be large; when smaller amounts are given, a greater percentage is retained. Acid feeding shows little effect on the retention of potassium—a diminution of only 8 per cent. In the alkali period, the potassium, instead of showing an increased retention, shows an actual diminution, only 70 per cent of that in the normal period being retained. Albu and Neuberg state the ratio of  $\frac{\text{Na}}{\text{K}}$  in the urine of an adult is 2.0

to 1.6. Table VII which gives the ratios of  $\frac{Na}{K}$ , shows that infants have an extremely large potassium intake in proportion to sodium and that this proportion in the intake is maintained in the urine, but not in the stool.

*Phosphorus.*—Probably next to calcium and in direct connection with it, the metabolism of phosphorus has received the greatest attention. The phosphorus intake in infancy (Table VIII)

TABLE VII  
The Ratios of  $\frac{Na}{K}$

Period	Intake	Excretion		Retention
		Feces	Urine	
I	0 60	1 26	0 65	0 27
II	0 60	1 18	0 99	
III	0 67	0 73	1 09	2 0
IV	3 04	1 75	3 50	

TABLE VIII  
Phosphorus

Period	Intake		Excretion						Retention		
			Feces			Urine					
	gm	cc	gm	cc	per cent	gm	cc	per cent	gm	cc	per cent
I	0 804	418	0 21	97	80	0 457	256	57	0 137	80	17
II	0 804	418	0 14	65	17	0 544	320	67	0 120	70	16
III	0 505	263	0 143	67	28	0 270	157	53 5	0 092	53	18
IV	0 505	263	0 180	84	36	0 260	150	51	0 065	38	13

is large in order to meet the requirements for growing bone. Milk is especially rich in phosphorus. For a 70 kilo man, the intake would amount to 6.3 and 6.4 gm of phosphorus per day. Excretion is largely by way of the urine—in the normal periods 68.5 and 65 per cent. That acid shifts the phosphorus from the stool to the urine is confirmed in our data; the phosphorus decreased 0.07 gm. in the stool and increased 0.087 gm. in the urine. The percentage of organic phosphorus in the stool we have arbitrarily, as the result of other experiments, calculated as 90 per cent of the

total phosphorus. On this assumption, acid causes a decrease of 32 cc. in the stool and an increase of 64 cc. in the urine. Under the influence of alkali the change is very small, but opposite, less being excreted in the urine and more in the stool.

The retention of phosphorus is very slightly diminished by both acid and alkali. The value of the phosphorus retained is difficult to estimate. Some must be stored as neutral phosphorus in phosphatides, nucleoproteins, and phosphoproteins. In calculating the acid value to the body, this quantity should be subtracted. However, if it should be burned to  $\text{PO}_4$  and excreted, it would again assume its value as an acid in base balance. A satisfactory approximation would probably be 90 per cent of the value as acid when burned and 80 per cent when stored.

TABLE IX

*Sulfur*

Period	Intake		Excretion						Retention		
			Feces			Urine					
	gm	cc	gm	cc	per cent	gm	cc	per cent	gm	cc	per cent
I	0 400	282		10	4	0 241 0 206	130	60	0 146		36
II	0 400	212		10	4	0 260 0 214	134	65	0 125		31
III	0 251	158	0 014	9	5	0 126 0 153	78	57	0 100	71	38
IV	0 251	133	0 019	12	8	0 132	83	62	0 79	38	30

*Sulfur*.—The sulfur values in our experiments seem large. For a 70 kilo adult, the intake would be 3.1 and 3.2 gm. of sulfur per day. Excretion is almost wholly by the urine—90 per cent. Acid and alkali increase the excretion slightly. Unfortunately, the analyses of the sulfur in the feces of the first two experiments were mislaid and arbitrary values have to be assigned. However, the values of sulfur in the feces are so small as to be practically negligible. In Blauberg's case they amounted only to 9 and 6 cc., and in Peiser's (2), to 8 cc. on breast milk. Retention is considerable, either as absolute or as percentage of intake, and varies inversely with excretion in the urine. It falls slightly when acid or alkali is given.

The acid value to be assigned to the sulfur stored is a problem similar to that of phosphorus, but concerns a larger amount. At least 50 per cent, probably more, of the sulfur stored is not neutralized by base. If the protein is calculated as 1 per cent sulfur, enough sulfur is retained for 8 to 10 gm. of protein, but since there are probably not more than 3 to 4 gm. of protein stored, half the sulfur can be utilized elsewhere. Chondroitin sulfuric acid and sulfatides also remove sulfur. What value shall be assigned to the remaining sulfur as  $\text{SO}_4$ ? Bones, blood, and tissue juices contain very little. Probably 85 per cent of the total sulfur has acid value as  $\text{SO}_4$  when burned, and 25 per cent when stored.

TABLE X.  
*Chlorine.*

Period	Intake		Excretion						Retention		
			Feces			Urine					
	gm	cc	gm	cc	per cent	gm.	cc	per cent	gm	cc	per cent
I	0 788	222	0 0135	4	2	0 651	184	83	0 123	32	15
II	1 650	464	0 302	85	18	1 615	455	96	-0 267	-75	-16
III	0 545	154	0 0182	5	3	0 454	130	85	0 063	18	12
IV	0 545	154	0 0485	14	9	0 528	148	97	0 020	-8	-5

*Chlorine.*—The intake of a 70 kilo adult, calculated from the figures in Table X, is 0.4 and 0.45 gm. of chlorine, or 6.5 to 7.5 gm. of sodium chloride, which would be considered moderate. Excretion is almost wholly by the urine, in our experiments 98 and 97 per cent. It is quite interesting and no doubt important, if confirmed, that when acid is given, chlorine may be excreted by the bowel. In Period II (acid) twenty times the normal is excreted—an actual increase of 80 cc. of 0.1 N Cl. When chlorine is given as HCl, practically all the intake is excreted in the urine. The bowel also excretes a large amount. The sum is a considerable negative balance of chlorine. Alkalies, also, cause an increase in the excretion of chlorides in the urine and feces and change a positive balance to a small negative balance.

## CONCLUSIONS.

The effect of acids and alkalies upon mineral metabolism shows many interdependences. Comparison of the various elements in terms of normal solutions is possible. Explanation is often obscure and until the facts have been firmly established do not warrant speculation. Each element is a problem in itself, but is also linked with the excretion and retention of others. The effect of acids and alkalies upon the elements studied can be summarized as follows:

1. In the urine, HCl increases the excretion of ash, nitrogen, calcium, sodium, phosphorus, and chlorine. Potassium, magnesium, and sulfur are not affected

2. In the feces, HCl increases the excretion of ash, nitrogen, calcium, magnesium, and chlorine. Sodium and potassium are not affected. It decreases the phosphorus.

3. In retention, HCl decreases ash, nitrogen, calcium, magnesium, sodium, potassium, phosphorus, sulfur, and chlorine.

4. In the urine,  $\text{NaHCO}_3$  increases the excretion of ash, nitrogen, sodium, potassium, sulfur, and chlorine. It decreases calcium, magnesium, and phosphorus.

5. In the feces,  $\text{NaHCO}_3$  increases the excretion of ash, nitrogen, calcium, sodium, potassium, phosphorus, sulfur, and chlorine. It decreases magnesium.

6. In retention,  $\text{NaHCO}_3$  increases the magnesium and sodium. It decreases the ash, nitrogen, calcium, potassium, phosphorus, sulfur, and chlorine.

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# URINARY EXCRETION OF ORGANIC ACID AND ITS VARIATION WITH DIET.

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The objects of this investigation were to determine the daily urinary excretion of organic acids by normal women on their customary diet, to see how the excretion of organic acid during the day while the subjects were engaged in their ordinary activities differed from that during the night while the subjects rested, and also to find how diet affected the excretion of organic acids during the day.

## *Review of the Literature.*

Until Van Slyke and Palmer reported (1) in 1920 their method of determining the total organic acids of urine, the methods were too long to be used frequently for the determination of a factor of unknown importance. They showed that during the development of acidosis the increase in the total organic acids of the urine runs nearly parallel to the increase in acetone bodies, one of the factors commonly used for diagnosing the stage of advancement of acidosis.

Their results have since been confirmed by others. Goiffon and Nepreux (2) found that, in general, in cases of acidosis the elimination of total organic acids in the urine was proportional to the elimination of  $\beta$ -hydroxybutyric acid, but that the curves showing rate of increase of each were less similar than they had been led to expect. They found cases in which there was increase in total organic acid excretion without simultaneous acetonuria; that is, they say,  $\beta$ -hydroxybutyric acid is not the only factor in acidosis though it is the most frequently occurring and the most important factor. Labbé, Bith, and Nepreux (3) approve of applying the method to a study of diabetic acidosis because in addition to showing increase in  $\beta$ -hydroxybutyric acid and acetoacetic acid it likewise takes into account lactic, butyric, succinic, propionic, capric, and other acids known to increase with the development of acidosis in a diabetic. Utheim (4), also using the Van Slyke-Palmer method for the determination of organic acid excretion, found that the urine of infants in a chronic con-

dition of extreme malnutrition showed most excessively high organic acid content compared with that of normal infants. This enormous excess, Utheim says, was certainly not due to acetone bodies nor lactic acid because, as he points out, whereas most of the common fatty acids, particularly those of the fatty acid series, are soluble in ether, he found that only 20 per cent of the organic acid of the urine of these infants was extractable with ether. Utheim attributed the increase as due largely to that of oxyproteic acids. The constitution of the oxyproteic acid fraction is not definitely known, but it is made up (5) of acidic substances precipitable by mercuric acetate and having barium salts soluble in water and insoluble in alcohol. Ginsberg says the oxyproteic acids may be polypeptides because on hydrolysis they give amino-acids. Freund and Sittenberger-Kraft (6) found that the amorphous barium salt, precipitated by alcohol, has no characteristic protein reactions and corresponded to an acid,  $C_{10}H_{22}O_{10}N_2$ . They thought it was probably a derivative of urea because it yielded this on hydrolysis. In contrast to Utheim's results on malnourished infants, Kohn (7), using von Furth's method (8) for the determination of oxyproteic acids and working with adults in a condition of chronic undernutrition from lack of sufficient food, found that there was a decrease in oxyproteic acids in the urine.

These uses of organic acid determinations for the study of metabolism under abnormal conditions suggest their use for investigations of normal metabolic processes. Labbé, Bith, and Nepreux (3), whose work on acidosis was referred to above, reported that in the course of their experiments they had found that changes in the diet effected changes in the elimination of total organic acid. Organic acid decreased on fasting and on a diet including green legumes, fats produced little increase, but there was an increase with the feeding of meat. No figures are given for these conclusions, but since corrections for organic bases were not made in their other determinations of organic acids, it seems that the increase on a meat diet may be due in part to the creatine of the food. Blatherwick and Long (9) found that in two normal young women the drinking of large amounts of orange juice in addition to a constant, creatine-free, basal diet brought about marked increase in organic acid excretion, an increase which they explained as due to part of the citric acid of the food which was not oxidized but was eliminated as citrate. They further reported that milk in which the organic acid content had been increased by inoculation with lactic acid bacteria to approximately 2 per cent, expressed as lactic acid, did not increase organic acid excretion above that found on a whole milk diet. Which of the organic acids are increased or decreased with change in conditions and diet, to what extent the change takes place, and what effects the change, are still interesting problems for solution.

Though little work has been done in the determination of total organic acids, many determinations have been made of individual organic acids in the urine, particularly in the study of certain diseases. The influence of diet on the output of a few of these acids has been determined to a limited extent. Acetone bodies, including  $\beta$ -hydroxybutyric acid and

acetoacetic acid, because of their strikingly increasing quantity in the urine in acidosis, have received much attention. According to the  $\beta$ -oxidation theory (10), an increased elimination of these substances is brought about by conditions that interfere with normal oxidative processes. In normal persons a diet lacking in carbohydrates, or, with low carbohydrate, produces increased excretion of these acetone bodies (11).

Amino-acid excretion, like that of acetone bodies, has been used in diagnosing the severity of diabetes mellitus and the increase explained as due to decreased oxidizing power of body cells. Increased protein ingestion under such abnormal conditions causes high amino-acid excretion (12).

Hippuric acid excretion in the urine of pellagrins, especially those on a corn-vegetable diet, was found to be two or three times as great as the quantity found in the urine of normal men on a mixed diet (13). Murlin says that since coarse vegetables contain, especially in the skins and raw fibers, salts of benzoic acid, he thought these might be set free in the intestines or that benzoic acid might be produced by the putrefaction of proteins in the presence of large quantities of carbohydrate. Also, Kohn (7) found that the urine of chronically undernourished individuals showed much higher hippuric acid than that of normal individuals (2.9 per cent against 0.66 per cent) and she explained this as due, perhaps, to the vegetarian diet of these undernourished individuals during the war. At least, an increase in hippuric acid excretion is indicated through the continued use of a diet low in adequate protein and high in coarse vegetables.

Mathews (14) says "Probably no nitrogenous substance in the urine has been more studied than uric acid." Its origin in the nucleoproteins of the food and of the body cells is well established and there seems to be agreement among investigators as to the composition of the intermediate products. A diet high in nucleoproteins, such as one consisting largely of glandular organs, increases the uric acid output (15), but the quantity of uric acid excreted has been found to vary with other factors of the diet. Mendel and Stehle, in their review of the literature and in their own experiments (16), bring out many facts to support the theory that the quantity of uric acid excreted is influenced by the stimulating effect of foodstuffs on the digestive glands. Folin (1905), Mendel and Brown (1907), Smetankamars (1911), Lambling and Dubois (1914), Maurel (1914), and Taylor and Rose (1914) showed that there was increased output of uric acid following ingestion of purine-free food. Especially was this found to be true of high protein meals (17). Lewis, Dunn, and Doisy (18), finding that small quantities (10 gm.) of amino-acids increased markedly the quantity of uric acid eliminated, put forward the theory that such increases were brought about through stimulation of cellular metabolism for they say the extent of nuclear breakdown necessary to account for the marked increase in uric acid in the urine would be far too great to be the result of stimulation of the cells of the digestive tract only. Lewis and Corley (19) found that fats and carbohydrates, in the amounts they fed, had no influence upon the excretion of uric acid.



Amberg and McClure, who together with Mayer, have in many ways identified citric acid in the urine without actually isolating the pure compound, say (20) that considering the citric acid found in milk it is not strange to find it in the urine, but that feeding experiments of Salant and Wise and their own experiments with the administration of sodium citrate led them to believe it improbable that the citric acid in urine comes from that in food. Blatherwick and Long (9) thought that the increase in organic acid output following the drinking of large quantities of orange juice was due to unused acid of the food, but they do not report the identification of the acid

Clogne and Fliessinger (21) advocate the use of glucuronic acid output determinations as a test of liver function. They found that in normal individuals glucuronic acid was often present in the urine during the 2nd hour after a meal, reached a maximum during the 3rd hour, and disappeared by the 4th hour after a meal accounting, thus, for its absence in some samples of urine. Tollens and Stern (22) found that feeding normal persons on meat alone with very little carbohydrate in the diet had no apparent effect on the excretion of glucuronic acid

Strisower (23) concluded that the amount of formic acid contained in normal urine is little influenced by either the diet or muscular activity. Dakin, Janney, and Wakeman (24) think that formic acid is partly of endogenous origin, an end-product of the metabolism of carbohydrates, of fats, and of proteins

#### EXPERIMENTAL WORK.

##### *Procedure.*

Eight students in the Nutrition Laboratory, six of whom were graduate students and the other two seniors in the Home Economics Department, served as subjects for this investigation. All were healthy women leading the ordinary sedentary life of college students, spending much of their time in the laboratory. Each was asked to keep a daily record of her diet during the days of collection of urine, but no restrictions were placed on the diet except that nothing be eaten between the evening and morning meals. Three, or, in one case several, approximately 24 hour samples of urine were collected by each woman, night and day samples being kept separate. The bladder was emptied at a definite time on arising in the morning; urine passed between that time and a definite time just before going to bed at night constituted the day sample and was preserved with toluene. Any urine passed during the night and that on arising in the morning constituted the night urine. Thus the night samples

were differentiated not merely on the basis of time as has usually been done, but by the fact that the subjects were in bed during the time covered, and then only. As soon as possible the next day, both samples were analyzed for organic acids and for creatinine.

### *Methods.*

Organic acids were determined by the Van Slyke-Palmer method (1). If, while titrating, much foam was formed during the shaking required for thorough mixing, a drop or two of ether was added to lower the surface tension. It was found that in cases of night urines where the volume was much smaller than for a corresponding number of hours during the day slightly higher values were obtained if the urine was first diluted with

TABLE I.  
*Effect of Dilution on Total Organic Acid in Night Urine.*

Subject	Date	Duration of collection	Volume of urine	Organic acid per hr , uncorrected	
				If undiluted	If diluted.
	1923	hrs	cc	cc 0.1 N	cc 0.1 N
M. L.	Apr 12	7 78	227	17.4	17.7
L. M.	Mar 30	7 9	282	13 2	13.4
L. M.	" 31	8 3	138	16 9	17 2

an equal volume of water. This procedure was therefore followed with most night urines and other less dilute samples. A few results are given in Table I. Higher results were to be expected if the solution was not sufficiently diluted to be considered completely ionized.

It was thought that creatinine was the only organic base present in sufficient quantity to affect the results markedly and it was the only one determined in the first experiments. Folin's microchemical method was used.

Data and results obtained on the total organic acids are given in Table II.

### DISCUSSION OF DATA.

Because the periods for collection of urine varied from 21 to 25 hours, the quantities of creatinine and organic acids, determined

in the samples of urine for each period, were calculated for comparison on the basis of a 24 hour period. The urine of eight normal women contained per day from 394 to 571 cc. of 0.1 N organic acids, uncorrected for organic bases. Blatherwick and Long (9), for two young women on creatine-free diet, obtained values of 499 and 548 cc. of 0.1 N acid. These values are comparable with those found by Van Slyke and Palmer (1) for ten

TABLE II  
*Daily Urinary Organic Acid Excretion of Normal Women.*

Subject	Weight	Date	Volume of urine	Creatinine	Organic acid			
					Uncorrected	Corrected	Uncorrected	Corrected
	kg	1923	cc	mg	cc 0.1 N	cc 0.1 N	cc 0.1 N per kg	cc 0.1 N per kg
L. M.	52.3	Feb. 12	1,305	1,132	505	405		
		" 13	1,555	1,354	571	451		
		" 18	1,295	1,074	570	476		
		" 20	1,615	1,110	498	392		
		" 21	1,335	1,187	573	468		
		" 26	1,604	1,225	515	407		
		Mar. 30	2,425	1,118	473	364		
		Apr. 1	2,566	1,162	490	392		
		Average		1,170	524	419	10.0	8.0
G. B.	54	Average.		1,070	475	380	8.7	6.9
K. D.	64	"		1,093	455	355	7.1	5.5
E. F.	58.1	"		1,039	394	307	6.8	5.2
R. G.	62.3	"		1,196	428	324	6.9	5.2
M. L.	69.9	"		1,553	571	443	8.2	6.3
I. S.	57.2	"		1,103	564	462	9.9	8.1
H. S.	60.2	"		1,209	423	317	7.0	5.3
Average .....				1,180	478	376	8.2	6.3

normal men. They obtained 412 to 748 cc. Goiffon and Ne-preux (2) got normal values of 300 to 450 and 500 cc. and Labbé, Bith, and Nepreux (3) say that, with the indicator used, the figures for 0.1 N organic acid range from 300 to 700 cc. per day. It will be noted that the range found for organic acids (uncorrected) for these eight women is 6.8 to 10.0 cc. of 0.1 N acid per kilo of body weight with an average of 8.2 cc. per kilo. For their

normal young men, Van Slyke and Palmer obtained a range of 5.7 to 9.7 cc., with an average value of 8.2 cc. per kilo of body weight.

*Variation in Urinary Organic Acids from Day to Day.*

The variation from day to day in organic acid excretion for a single individual was considerable though there was no striking difference in the daily consumption of food so far as kinds and amounts were concerned. The greatest difference shown was for subject I. S., with a difference between the highest and lowest values of 139 cc. of 0.1 N organic acid (corrected), a percentage difference of approximately 30 per cent. For one subject, K. D., the 3 days of this experiment formed the preperiod of a coefficient of digestibility experiment and the food for each day was weighed out so as to be the same on all days. Consisting of meat, potatoes, bread, butter, milk, apples (477 gm.), and sugar, it contained 60.2 gm. of protein, 62.9 gm. of fat, and 241.2 gm. of carbohydrate and had an energy value of 1,772 calories. The average organic acid excretion for the 3 days was 355 cc. of 0.1 N acid (corrected) with a difference between the highest and lowest values of 80 cc., a percentage difference of approximately 23 per cent. It is apparent that diet is not the only factor determining organic acid excretion.

*Variation in Urinary Organic Acids with Day and Night.*

Day and night urines were analyzed separately because it was thought that to a large extent any immediate influence of muscular activity and to some degree the effect of diet were eliminated from metabolic processes involved during a night period limited to the time while the subject was in bed and beginning 3 to 5 hours after eating. The results are tabulated in Table III for comparison of day and night urines.

On the average and in individual cases with one exception the organic acid excretion per hour was markedly higher during the day periods than during the night. In the exceptional case the results for the two periods were the same. However, this subject unlike any of the others ate an apple just before going to bed the first night of the experiment and ate both an apple and

an orange the other two nights. This distinctly higher day excretion is contrary to the findings of Van Slyke and Palmer who say (1): "There appears to be little difference between day and night periods in rate of organic acid excretion." However, the figures they give are for hospital cases and the 24 hour days were divided into two equal 12 hour periods, from 6 o'clock until 6 o'clock. If normal muscular and nervous activity and if

TABLE III  
*Day and Night Excretion of Organic Acids in the Urine.*

Subject	Date	Volume of urine	Period	Creatinine per hr		Organic acid per hr	
				Day	Night	Day	Night
	1923	cc	hrs.	mg	mg	cc 0 1 N	cc 0 1 N
L. M	Feb. 12	915	15 33	48		21	
	" 12	390	8 5		46		10
	" 13	1,290	15 67	56		21	
	" 13	265	8 5		56		12
	" 18	215	8 0		43		10
	" 19	1,080	16 0	46		25	
	" 20	240	7 75		40		10
	" 21	1,375	16 25	49		19	
	" 21	365	8 25		44		15
	" 22	970	15 75	52		22	
	" 26	537	8 25		53		14
	" 27	1,067	15 75	50		19	
	Average			50	47	21	12
G. B	Average			46	43	18	13
K D	"			47	42	17	12
E F.	"			40	43	14	12
R G.	"			54	45	16	10
M. L	"			61	62	22	12
I S	"			44	45	19	19
H S	"			53	45	15	9
Average				49	47	18	12

diet affect the organic acid excretion it was hardly to be expected that there would be similarity between these two "night" periods, the one that of normal individuals and covering a period of relative inactivity compared with that of their day, and, the other that of hospital cases with a night period beginning sooner after meals and differing comparatively little from the day period as far as muscular activity was concerned.

*Effect of Diet on Urinary Organic Acids.*

*Procedure.*—By the analysis of many hourly samples of urine of one subject it was found that there was much more striking variance in organic acid excretion from hour to hour during the day than there was in the creatinine output. The subject was working in the laboratory most of each day so that it was thought that the greatest factor causing variation might be food. To find the effect of diet, experiments were carried on according to the following procedure. The same subject, a woman 5 ft. 2.6 in. tall and weighing 52 kilos, was used throughout the experiments. In one series of experiments eight or nine hourly samples of urine were collected on the experimental days, the subject drinking about 200 cc. of water each hour and carrying on routine laboratory work throughout the day. Each day the first two to four samples of urine were collected before a weighed creatine-free meal was eaten. On each day of another series of experiments a sample of urine was collected before the subject ate a weighed creatine-free meal and then two 2 hour samples of urine were collected. During the days of the experiments the hourly excretion of total organic acids, corrected for creatinine, was determined by analyses of the samples of urine. Legal's nitroprusside test for acetone carried out repeatedly on the samples of urine did not give a positive reaction. Rothera's modification of this test was applied to the first few cubic centimeters of distillate from many samples of urine. The development of a faint lavender ring indicated traces of acetone. In no case was the reaction positive enough so that it might be considered worth while to determine acetone bodies or to feel that their presence interfered with the color determination of creatinine.

*Organic Acids in Fasting Samples of Urine.*—In twenty-three determinations on samples of urine collected during fasting the "normal level" of total organic acid excretion for the subject was found to be variable within limits, with an average of 18 cc. of 0.1 N acid and a range of 15 to 24 cc. of 0.1 N acid per hour. Though in all cases no food had been eaten for 12 to 15 hours this variance in organic acid output might be thought directly due, at least in part, to previous diet were it not that the organic acid content of the night urine, determined

TABLE IV.

*Effect of Fruit, Low Protein Diet on Urinary Excretion of Organic Acid.*

Date.	Time of meal	Time of collection.	Volume.	pH	Creatinine per hr.	Organic acid per hr., corrected.
<i>1935</i>		<i>a m</i>	<i>cc</i>		<i>mg.</i>	<i>cc. 0.1 N</i>
Mar. 28	11 45 a.m.	8	94	6 8-7.0	55	17
		9	240	6 8-7.0	56	15
		10	212	6 8	51	17
		11	285	6 8	52	15
		<i>p m.</i>				
		12	112	6 8-7 0	53	16
		1	420	6 4	50	22
		2	105	6 6-6 8	55	22
		3	208	6 6	55	25
		4	310	6 4-6 6	49	25
Mar. 30	12.10 p m.	<i>a m</i>				
		9	132	5 0	58	18
		10	474	6 4	52	21
		11	370	6 6	48	19
		<i>p m</i>				
		12	64	5 0-5 2	51	22
		1	220	5 6-5 8	47	17
		2	265	5 6-5 8	50	23
		3	165	5 2	52	25
		4	223	5 0-5 2	50	23
Apr. 1	11.55 a m.	<i>a m</i>				
		9	208	6 8-7 0	58	17
		10	138	6 8-7 0	57	17
		11	370	7 0	55	19
		<i>p m</i>				
		12	205	7 0	53	15
		1	192	6 2-6 4	48	17
		2	172	6 2-6 4	48	21
		3	206	6 2-6 4	52	28
		4	286	6 0-6 2	49	24
		5	220	5 2	47	21
June 25	10.00 a.m.	<i>a m.</i>				
		10	200		48	17
		<i>p m</i>				
		12	318		47	20
July 2	9.00 a.m.					
		<i>a m</i>				
		9	280		49	14
		11	297		49	18
		<i>p.m</i>				
		1	237		51	25

many times for this subject, was always lower, varying from 10 to 15 cc. of 0.1 N acid per hour, corrected for creatinine. Either there is some stimulation of the cell on awaking or substances held in the tissues during the night are eliminated in the morning or else the activity of waking hours brings about a speeding up of processes. The last hypothesis would explain the highest results, those obtained on March 30. During that morning the

TABLE V.

*Effect of Low Protein Diet on Urinary Excretion of Organic Acid.*

Date	Time of meal.	Time of collection	Volume	pH	Creatinine per hr.	Organic acid per hr., corrected.
<i>1928</i>		<i>a m.</i>	<i>cc</i>		<i>mg</i>	<i>cc 0.1 N</i>
May 19	10.45 a.m.	8 29	159	4 5	61	16
		9.31	123	5 3	59	16
		10 33	168	5 3	60	17
		11 33	272	5 3	56	17
		<i>p m</i>				
		12 35	59	4 9	59	19
		1 35	163	4 5	60	18
		2 36	302	4 5	57	17
		3 36	201	4 5	53	15
June 18	10.00 a.m.	<i>a m</i>				
		10 00	463		54	20
		<i>p m</i>				
		12 00	627		50	23
June 21	9 00 a.m.	2 00	235		52	22
		<i>a m</i>				
		9 00	395		48	18
		11 00	373		47	22
		<i>p m</i>				
		1 00	81		45	19

subject attempted other work besides routine laboratory work and thus was forced to increased activity, especially during the hour just before noon.

*Content of Meals.*—In our experiments three types of weighed meals were used. The composition and energy content of each meal was calculated, using Bulletin 28 of the Department of Agriculture. The first meals consisted of bread, butter, milk, and a salad of lettuce, apples, dates, and nuts with a mayon-



## Urinary Organic Acids

TABLE VI.

*Effect of High Protein Diet on Urinary Excretion of Organic Acid.*

Date.	Time of meal	Time of collection	Volume	pH	Creatinine per hr.	Organic acid per hr., corrected.
1923		a m	cc		mg	cc 0.1 N
Apr. 21	10 30 a.m.	7 42	64	6 45	63	18
		8 42	220	6 25	59	18
		9 45	165	6 25	59	20
		10 45	295	6 25	58	23
		11 49	262	5 9	56	29
		p m				
		12 49	140	6 45	61	29
		1 49	61	6 8	56	26
		2 49	110	6 3	57	24
		3 49	235	6 0	55	25
Apr. 28	9 30 a.m.	a m				
		8 23	132	5 0	45	16
		9 24	232	5 9	54	17
		10 24	217	6 0	62	24
		11 27	345	6 2	60	29
		p m				
		12 27	220	6 4	59	33
		1 27	61	6 45	56	25
		2 27	187	5 9	53	24
		3 27	72	5 3	51	21
May 12	10 00 a m	a m				
		7 57	66		55	17
		8 57	232		58	23
		9 57	363		50	24
		10 59	126		59	26
		11 59	161		53	26
		p m				
		12 59	50		54	29
		2 00	253		53	30
		3 00	160		55	24
June 27	9 00 a.m	a m				
		9 00	537		51	18
		11 00	318		50	23
		p m				
June 29	9 00 a.m.	1.00	190		52	24
		a m				
June 29	9 00 a.m.	9 00	310		48	17
		11 00	328		48	23
		p m				
		1 00	196		48	26

naise dressing. Each contained 20.8 gm. of protein, 70.6 gm. of fat, and 125.5 gm. of carbohydrate and had an energy value of approximately 1,220 calories. About 60 per cent of the carbohydrate content was from fruit, indicating relatively high organic acid in the food. Fruit was excluded from the second type of

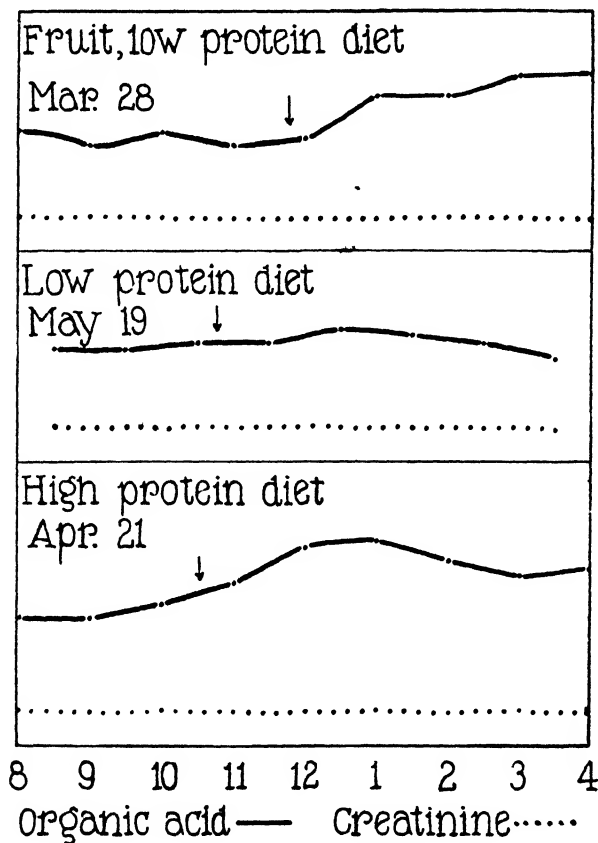


FIG. 1.

meal which contained approximately the same weights of protein, fat, and carbohydrate. It consisted of bread, butter, a glass of milk, and a cream pudding thickened with flour. The third type of meal was high in protein and consisted of bread, butter, milk, eggs, cottage cheese, and nuts or peanut butter. The

quantity of protein varied from 54 gm. on 1 day to 68 or 69 gm. on 4 days with a variation in energy content from 775 to 1,040 calories. See Tables IV, V, and VI, and Fig. 1.

#### DISCUSSION OF DATA.

After the low protein, non-fruit diets the increase in organic acid excretion was small; that is, if organic acids were formed as products of the intermediate metabolism of 20 gm. of protein, 72 gm. of fat, and 125 gm. of carbohydrate, oxidation of most of them was effected before elimination from the body. After the fruit salad meals and also after the high protein meals distinct increase in organic acid excretion resulted. The effects are especially pronounced when comparison is made with the creatinine excretions calculated in cubic centimeters of 0.1 M solutions. The effect of the high fruit diets reached a maximum in 3 hours. The period of maximum effect of the high protein diets varied from 2 to 4 hours, depending perhaps upon the energy content of the meal and state of division of the food.

#### *Comparison of Effect of Diet and Effect of Activity.*

The increase in urinary organic acid excretion due to high fruit or high protein diets was, likewise, marked when compared with the variation found earlier in the day before food was eaten. Though the volume of 0.1 N organic acid (corrected for creatinine) in hourly samples of urine collected while the subject fasted varied from 15 to 24 cc. still after diets high in fruit or in protein the volume of 0.1 N organic acid rose to 28 to 30 or to 33 cc. That is, in some cases, due to diet, the quantity of organic acids eliminated in the urine per hour was nearly doubled.

The hourly excretion of organic acids during the night for this subject varied from 10 to 15 cc. of 0.1 N acid. The difference between this and the output while fasting and working when compared with the difference between the fasting level and the maximum hourly excretion after a high fruit or a high protein diet shows that such diets affect the rate of elimination of organic acids to approximately the same extent as does ordinary activity.

*Individual Acids Affecting Total Organic Acid Excretion.*

What acids constituted this increase was a question. Calculations made from figures given in the literature for average urinary excretion of special organic acids indicated that the acids likely to be present in the greatest amount, calculated in terms of a normal solution, were uric, amino-, hippuric, and citric acids. Taylor and Rose (17) and Lewis and Corley (19) found that uric acid elimination was increased by protein feeding while fat and carbohydrate in the amounts consumed, amounts comparable with those of the experiment here reported, had no effect on uric acid output.

*Uric and Amino-Acids.*—To determine what fractions of the organic acid output and what fractions of the increase in organic acid output were due to uric acid and to amino-acid these factors were determined in fasting samples of urine and in the two 2 hour samples of urine collected after eating each of the three types of meals used in our experiments. A Folin colorimetric method was used for each (25).

After the low protein meals uric acid output remained about the same as it was before eating or decreased as it has been found to do in the morning hours during fasting (19). After fruit salad meals and also after high protein meals, uric acid output was increased. It seems that some substances present in fruit may, like products of protein digestion, stimulate the metabolic processes that bring about production of uric acid as an end-product, as they did for total organic acids. On 1 day (3rd day of that series) when a high protein meal was eaten, uric acid was determined hourly and found to increase at about the same rate as total organic acids, but whereas the total organic acids continued to increase for about 4 hours uric acid output reached a maximum in 2 to 3 hours.

After the low protein, non-fruit meal no apparent change was produced in amino-acid elimination. There was slight increase after the fruit salad meals and a more marked increase after the high protein meals. However, if for purposes of calculation, the amino-acids are considered to be monoamino-monocarboxylic acids and the quantities present in the urine were calculated in cubic centimeters of 0.1 N solution, amino-acid elimination was

found to be very low in all cases. And, especially, as only 40 per cent is titrated in the Van Slyke-Palmer method, amino-acids formed a small fraction of the total organic acids determined in the urine.

TABLE VII  
*Urinary Excretion per Hour of Organic Acids and Bases.  
Variation with Diet.*

Date, 1923	June 18	June 21	June 25	July 2	June 27	June 29
Diet	Low protein. 19 gm protein		Fruit salad 21 gm protein		High protein 69 gm protein	
Fasting.						
Organic acid (uncorrected), cc 0.1 N	25	22	21	18	23	21
Creatinine, cc. 0.1 M	4.7	4.3	4.2	4.3	4.5	4.2
Creatine, * titrated, cc 0.1 M	0.5	0.2	0.2	0.3	0.2	0.2
Uric acid, † cc 0.1 N	3.2	3.0	2.6	3.1	4.1	4.2
Amino-acid, ‡ titrated, cc. 0.1 N		1.3	0.9	0.9	1.4	1.4
First sample after eating						
Organic acid (uncorrected), cc 0.1 N	28	26	25	23	27	27
Creatinine, cc 0.1 M	4.4	4.1	4.2	4.3	4.4	4.2
Creatine, * titrated, cc 0.1 M	0.5	0.2	0.2	0.3	0.4	0.4
Uric acid, † cc 0.1 N	2.9	3.1	3.6	4.3	4.5	4.7
Amino-acid, ‡ titrated, cc. 0.1 N		1.4	1.3	1.1	2.1	1.6
Second sample after eating						
Organic acid (uncorrected), cc 0.1 N	27	23	30	29	29	30
Creatinine, cc 0.1 M	4.6	4.0	4.4	4.5	4.6	4.3
Creatine, * titrated, cc 0.1 M	0.4	0.6	0.3	0.2	0.5	0.5
Uric acid, † cc. 0.1 N	2.5	3.1	2.7	4.8	5.1	5.1
Amino-acid, ‡ titrated, cc. 0.1 N...		1.4	1.0	1.1	2.2	1.6

\* Volume, calculated from weight determined, was multiplied by 0.60, the fraction of it titrated in the Van Slyke-Palmer method

† Volume calculated, considering uric acid as a tribasic acid

‡ Volume, calculated from weight of amino-acid nitrogen determined, multiplied by 0.40, the fraction titrated. For calculation, acids were considered as monoamino-monocarboxylic acids.

Inspection of Table VII makes it evident that in the fraction of urine titrated in the Van Slyke-Palmer method, creatinine and uric acid are important factors, that, at least in one subject, creatine is not to be disregarded, and that when one considers

the great number of individual organic acids present, amino-acids account for a fraction that must be considered since the portion of it titrated composes more than 5 per cent of the total volume titrated. These four compounds make up one-third to one-half of the organic acids and bases titrated in these experiments.

#### SUMMARY.

1. The daily urinary excretion of organic acid by eight normal women on their customary diets was determined by the Van Slyke-Palmer titration method. The values obtained, 6.8 to 10.0 cc. (uncorrected) of 0.1 N acid per kilo per day, were the same as those found for men by other investigators.

2. The variation from day to day in organic acid output was considerable even when the diet was constant.

3. Day periods when the subjects were engaged in their customary activities showed much greater excretion of organic acid per hour than did night periods limited to the time that the subjects spent in bed.

4. The hourly excretion of organic acids during fasting varied from 15 to 24 cc. of 0.1 N acid.

5. A high calory meal in which only 6 to 7 per cent of its energy content was due to protein had little effect on organic acid excretion.

6. Diets high in fruit or in protein cause markedly increased organic acid elimination. This increase is comparable with that produced by the activities of the day time.

7. Uric acid excretion was found to be increased by the same factors in the diet as those affecting total organic acid excretion.

8. Creatinine, creatine, uric acid, and amino-acids make up one-third to one-half of the quantity of organic acids and bases titrated in the Van Slyke-Palmer method.

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## SOME OBSERVATIONS ON THE CREATININE EXCRETION OF WOMEN.

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During the course of experiments on the urinary excretion of organic acid by women (1) creatinine determinations were made. The data show the daily creatinine excretion of eight normal women.

### *Review of the Literature.*

Folin's colorimetric method for creatinine has been used for almost 20 years so that many data have been collected. The creatinine excretion of individuals has been found to be "constant" so that some experimenters now use it in the determination of other components of the urine, where the time interval for collection is short, as a measure of the completeness of the emptying of the bladder. So confirmed are many investigators in their belief that muscular development, tonus, or efficiency is the most important factor in determining creatinine output that the creatinine coefficient (creatinine excretion expressed in milligrams of creatinine nitrogen per kilo of body weight) has been suggested as a measure of muscular development. Shaffer (2) in 1908 calculated for comparison with his own, data given by Folin, van Hoogenhuyze and Verploegh, Clossen, Osterberg and Wolf, and Klercker for the average daily excretion of normal men and found that the creatinine coefficient varied from 5.4 to 11.7 mg. These he takes as normal limits, but says that he believes that coefficients below 7 mg. are found only in abnormal cases such as elderly, excessively fat or inactive, poorly developed subjects. Creatinine coefficients for women reported in the literature are tabulated in Table I.



It is not surprising to find such a wide range for creatinine coefficients among normal subjects when one considers that the daily creatinine excretion for a single individual varies within limits showing a percentage difference frequently of more than 25 per cent. The term "constant" excretion is a relative one applicable, for instance, for comparison with other urine components, such as urea or even total organic acids. The hourly excretion of creatinine has been found to be variable to about the

TABLE I  
*Creatinine Excretion of Normal Women.*

No of subjects	Creatinine coefficients *			Investigator
	Minimum	Maximum	Average	
2	7 7	8 3	8 0	Shaffer (2).
3	4 8	7 4	5 8	Krause (3).
9	5 2	8 2	6 7	Palmer, Means, and Gamble (4).
26	3 5	9 8	5 8	Tracy and Clark (5).
6	4 3	5 6	4 9	Hull (6)
3	3 9	5 0	4 5	" (6).
2	5 2	7 9	6 1	Denis and Minot (7).
2	6 7	8 1	7 4	Rose, Dimmitt, and Bartlett (8)
2	6 7	6 9	6 8	Stearns and Lewis (9).
10	6 3	9 8	8 0	Dye (10).

\* In order to express all results in mg of creatinine N per kilo of body weight, recalculations were made by the authors from data given by investigators.

same extent as the daily excretion. Klercker (11), for example, found it to vary on single days in one individual from 60 to 76 mg. and from 68 to 83 mg. per hour and Neuwirth (12), fasting during the days of his experiments, showed variations on single days from 41 to 59 mg., 54 to 67 mg., and 50 to 81 mg per hour. That changes in muscular tonus from day to day or hour to hour can bring about such marked difference in creatinine excretion in a single individual hardly seems plausible. Schulz (13), from his experiments, concludes that muscular activity increases the amount of creatinine eliminated during the period of work, though it does not affect the total daily output. Campbell and Webster (14) found a distinct increase in creatinine after muscular activity only when the strain was severe so that they could explain it as due to damage

to the protoplasm. Van Hoogenhuyze and Verploegh (15), likewise, found that strenuous work did not affect creatinine excretion, but concluded that an increased elimination took place when the body was forced to live at the cost of its own tissues. According to Weinberg (16) muscular tonus is not even an important factor. Using as subjects hospital patients with normal muscular tonus and no disease thought to affect creatinine metabolism, he found extremely low creatinine coefficients, varying from 2.1 to 3.0 mg. Patients with increased muscular tonus and two with hypotonic muscles, each gave an average coefficient of 4.3 mg., indicating that muscular tonus was not the determining factor. Subjects from an insane hospital, chosen because they were cases with preoccupied or diffused consciousness, showed higher creatinine coefficients, varying from 4.3 to 6.9 mg. Weinberg says that previous investigators got high values for normal subjects because these subjects were students and so continuously preoccupied. His theory is that the mind affects creatinine excretion. What, then, determines creatinine excretion, it seems, is not yet settled. Comparison of day and night excretion does not show consistent results in all cases and comparison of the results of various investigators is difficult because of differences of method of limiting the time for the collection of night urine. Our results add a few figures.

#### EXPERIMENTAL WORK.

##### *Procedure.*

Creatinine was determined by the Folin method (17). The creatinine used as standards was obtained from the Special Chemicals Company, Highland Park, Illinois. The picric acid was purified by the method of Folin and Doisy (18). The tables for organic acid excretion show the creatinine excretion of these normal women. Averages are repeated in Table II and creatinine coefficients are given.

#### DISCUSSION OF DATA.

Attention is called to the creatinine coefficients with a range of 6.3 to 9.6 mg. of creatinine nitrogen per kilo of body weight and an average of 7.5 mg. The range Dye obtained for subjects living under the same conditions as our subjects was practically

the same, 6.3 to 9.8 mg.; her average was slightly higher, 8.0 mg. for her group of ten women. The range found is well within that calculated by Shaffer from his own data and that of many other investigators working with men. The variations for the different women are in accord with what might be expected on the basis of the theory that creatinine output varies with the mass of active protoplasmic tissue. K. D. and E. F., the shortest women and also with the greatest fat deposits, as indicated by appearance and positive deviation from the standard weight, also showed the lowest creatinine coefficients. It was especially interesting to compare G. B., R. G., and H. S. who show nearly the same

TABLE II  
*Daily Excretion of Creatinine in Urine of Normal Women.*

Subject	Weight.	Deviation from standard weight *	Creatinine	Creatinine N per kg.
	<i>kg</i>	<i>per cent</i>	<i>mg</i>	<i>mg</i>
G. B.	54.9	-5.7	1,077	7.3
K. D.	64.0	+12.6	1,093	6.3
E. F.	58.1	+7.4	1,039	6.8
R. G.	62.3	-4.9	1,196	7.1
M. L.	69.9	+5.3	1,553	9.6
L. M.	52.3	-9.4	1,170	8.4
I. S.	57.2	+5.0	1,103	7.2
H. S.	60.2	+6.0	1,209	7.0
Average				7.5

\* Standard of life insurance companies published in the Medico-Actuarial Mortality Investigation, 1912, 1, 66; reproduced in Blunt, K., and Bauer, V., *J. Home Econom.*, 1922, xiv, 1.

coefficient. H. S. was the most vigorous of the three, but this characteristic was counterbalanced by her being a few per cent overweight, while the other two were a few per cent underweight judged by life insurance standards. M. L., the largest woman, produced the greatest quantity of creatinine and her higher creatinine coefficient may be explained by the fact that she was an especially active, vigorous woman in the best physical condition. L. M., the other subject with a high creatinine coefficient was 9 per cent under the standard weight, a deviation from the standard greater than that of any of the others in the group. Underweight, in this case, may indicate only absence of the

usual fat deposits in the body and if so, a relatively high creatinine excretion was to be expected because of the higher percentage of active cells.

In order to study the relation between underweight and creatinine excretion ten young women, who were 15 or more per cent underweight, were used as subjects. Two samples of the night urine of each subject were analyzed within a few hours after collection. Night urine was defined in the same way as for the normal women used in the experiments. No food was eaten after the evening meal. The bladder was emptied just before going to bed and the urine collected up to the time of and including that on arising in the morning. Though Legal's nitroprusside test was applied to each sample of urine no positive test for acetone bodies was obtained. The creatinine coefficient for underweight women appears to be much higher than for normal women; in most of the cases studied the value found was above the range indicated for normal women.

Comparison of the day and night excretions of creatinine per hour shows no constant difference. For five subjects the average for day excretion was slightly higher whereas the reverse was true for three individuals. The average for all was 49 mg. of creatinine per hour by day and 47 mg. per hour by night.

The hourly creatinine output for one subject was determined for 8 hours on 7 days and plotted on the same scale as that used for organic acids. On these curves the creatinine output appears constant through the day, but when the figures for this excretion are compared with each other a fluctuation is quite marked from hour to hour through the day. A percentage difference between the minimum and maximum results for 8 hours of a day of from 13 to 21 per cent seems considerable. In our data incomplete emptying of the bladder is not indicated in the low creatinine values for they are not preceded nor followed by especially high values. The term, "constancy of creatinine output," as applied to hourly, or for that matter to daily, samples of urine is a very relative term.

*Creatine.*—In our experiments creatine was determined in hourly samples of urine during 3 days and in bihourly samples of 6 days, using Folin's colorimetric method (17). Though no creatine was found the 1st hour of 1 day, quantities varying from

4 to 24 mg. per hour were found to be excreted during the other hours of that day and of the other 8 days when tests were made. The results obtained in this series of experiments indicate no relation between high protein diet and creatine excretion.

#### SUMMARY.

1. The range for the creatinine coefficients of the normal women subjects was found to be well within the range found by Shaffer for men. Little difference was shown between the average day and the average night excretion of creatinine. The creatinine excretion of underweight women is greater than that of normal women.

2. Creatine was found to be an almost constant component of the day urine of one healthy woman.

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## UNIFORMITY IN INVERTASE ACTION.\*

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In their paper on "Uniformity in invertase action," Nelson and Hitchcock (1) found that six out of eight preparations of invertase studied, showed a uniform behavior with respect to the rate of inversion of a 10 per cent sucrose solution. In other words, the hydrolysis in each case conformed to the same empirical equation, involving time and per cent hydrolyzed. These six were Preparations 1, 2, 7, 8, A, and B. The remaining two, Nos. 3 and 6, showed a progressive decrease in velocity. Not only did these two differ from the majority but also from each other, and because of their apparent irregularity the authors termed them "abnormal" preparations.

A similar case of abnormality has been described recently by Willstätter, Graser, and Kuhn (2). They observed that all but one of several very active preparations gave the usual increasing monomolecular velocity constants during the course of the hydrolysis. This one, Preparation 1, gave practically constant coefficients under the same conditions.

Nelson and Hitchcock, and also Willstätter and his associates were of the opinion that the abnormality could hardly be due to an inactivation<sup>1</sup> of the enzyme occurring during the process of hydrolysis. The basis for this conclusion was the same in both cases: when the abnormal invertase preparation was permitted to remain in the constant temperature bath for the same length of time as that over which the hydrolysis was followed, no appreciable loss in activity was found to have occurred.

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<sup>1</sup> The term inactivation as used here refers specifically to the progressive loss of activity which invertase manifests on standing, especially at higher temperatures and acidities.

These experiments, however, do not take into account the possible influence of the hydrogen ion concentration on the rate of inactivation. That this factor is an extremely important one has been shown by O'Sullivan and Thompson (3), Hudson and Paine (4), and by von Euler and Laurin (5). Due to this omission the conclusion reached, that abnormality could not be due to inactivation, becomes questionable. The present paper, therefore, deals chiefly with a reexamination of this point.

The attempted inactivation of Preparation 3 by Nelson and Hitchcock was carried out at pH 5.8, without the addition of a buffer mixture. Since the abnormality was observed in the course of a hydrolysis at pH 4.5, it seemed more logical to the present investigators to look for inactivation in the neighborhood of this latter acidity. Under these conditions, a loss of activity was actually found to occur. In addition to Preparation 3, Nos. 6b, 7, and 8 were also studied; the two latter being normal preparations as shown by Nelson and Hitchcock. Unfortunately, none of No. 6 (one of the two original abnormal preparations of these authors) was available, and so No. 6b, No. 6 which had been dialyzed, was used instead of it.

Briefly, the procedure employed was as follows: Enough of a citrate buffer solution yielding the desired pH was added to the enzyme solution so as to be present in 0.01 M concentration. At the end of each of the indicated time intervals after mixing, a sample of this incubation mixture was withdrawn and added to a sucrose-buffer solution, such that the resulting hydrolysis mixture contained 10 per cent sucrose and buffer in 0.01 M concentration at a pH of about 4.8 at 25°C. Under these conditions the empirical equation of Nelson and Hitchcock

$$n \ t = \frac{\log 100}{(100 - p)} + 0.002642 \ p - 0.00000386 \ p^2 - 0.0000001034 \ p^3$$

is applicable. Taking three samples during the course of each hydrolysis, it is possible to calculate  $n$ , the velocity coefficient in the above relation. In this way, the activity at any time is determined as the average of three values of  $n$ , thus increasing the certainty of the results. The experimental details are given in the next section. Duplicate experiments were entirely independent.

Table I contains a summary of these experiments; the more detailed data are given in Table VI. As was anticipated, the two normal preparations show no decrease in  $n$  and, therefore, no loss of activity whatever during the period of incubation. Nos. 3 and 6b, however, show decreases of 1.1 and 2.7 per cent, respectively, during the 5 hour period, the length of time over

TABLE I

Experiment No.	Invertase No	Inactivation time	Varied condition.	$n \times 10^5$	Inactivated.
		<i>hrs</i>	<i>pH</i>		<i>per cent</i>
18a	7	0	4.8	823	
b		5		820	0.4
2b	8	2	4.8	988	
d		6		988	0.0
69a	3	0	4.9	840	
b		5		831	1.1
70a	3	0	4.9	840	
b		5		831	1.1
71a	6B	0	4.9	72.7	
b		5		70.7	2.7
72a	6B	0	4.9	72.7	
b		5		70.7	2.7
63a	3	0	+Invert sugar	801	
b		5	4.9	786	1.9
64a	3	0	+Invert sugar	802	
b		5	4.9	787	1.9

which the original abnormal hydrolyses were followed. Such an inactivation occurring during the course of a hydrolysis would obviously manifest itself by progressively decreasing velocity constants. It has been claimed by O'Sullivan and Tompson (3) and by Hudson and Paine (4) that both sucrose and fructose can exert a protective action on this inactivation, although its extent varies with the pH and the concentration of added carbohydrate. In view of this it becomes necessary to show that the sugars present during the hydrolysis are not able completely



to stabilize Preparation 3 over this period. Otherwise there remains the possibility of the observed decrease in  $n$  being due to some other cause, in spite of the inactivation which occurs in the absence of the sugars. Accordingly, Experiments 69 and 70 (Table I) were repeated with this modification, that invert sugar was present in the buffer solution before the invertase was added to give the incubation mixture. The concentration of invert sugar after completed inversion was approximately the same as

TABLE II

Experiment No	Invertase No	Inactivation time	Varied conditions	$n \times 10^4$	Inactivation.
		<i>hrs</i>			<i>per cent</i>
22a	3	0	+Boiled preparation No 8	856	0 0
b		5		856	0 0
47a	6B	0	+Boiled preparation No 8.	76 2	
b		5		74 1	2 8
48a	6B	0	+Boiled preparation No 8.	76 0	
b		5		74 1	2 5
23a	3	0	+0.02 M NaCl	790	
b		5		791	0 0
43a	6B	0	+0.1 M NaCl	75 8	
b		5		72 6	4 2
44a	6B	0	+0.1 M NaCl.	76 1	
b		5		72 4	4 9

that occurring in the hydrolyses described by Nelson and Hitchcock. A comparison of Experiments 63 and 64 with Nos. 69 and 70 indicates the absence of any such protective action.

With respect to the influence of added substances, Nelson and Hitchcock found that they were able to vary the degree of abnormality in the following ways: (1) by the addition of invertase which had been inactivated by boiling, and (2) by the addition of sodium chloride. If, now, inactivation is the cause of abnormality, then it should be similarly affected by these substances. This was found to be so. The results of these experiments are given in Table II while Table III contains a summary of the

observations of Nelson and Hitchcock. It must be remembered here that 0.7 per cent was arbitrarily fixed by these investigators as a boundary value of the A. D.<sup>2</sup> for the distinction of normal and abnormal preparations.

The procedure was the same as that described above except that in each case the indicated stabilizing agent was mixed with the buffer solution before the addition of the invertase. The inactivation of the added yeast extract was accomplished as

TABLE III.

*A Summary of Nelson and Hitchcock's Experiments with Abnormal Invertase.*

Experiment No (Nelson and Hitchcock)	Invertase preparation No	Invertase per 100 cc of solution	Varied condition.	A D
		cc		per cent
12	3	1 905	None.	1 4
17	6	10 45	"	1 9
20	7	3 60	"	0 56*
22	6B	16	Dialyzed and diluted.	1 8
25	3	1 905	+10 cc of boiled invertase 8.	0 34*
26	3	1 943	+10 " " " " 8.	0 40
35	6	10 45	+10 " " " " 8	1 4
44	3	1 905	+0 02 M NaCl	0 71*
42	3	1 905	+0 1 " "	0 46*
48	6	10 45	+0.1 " "	1.7
58	3	0 5	Concentration of invertase	2.7
54	3	3 0	" " "	0 74
56	3	6 0	" " "	0 67*

\*These hydrolyses are normal since the boundary A D value set for the distinction of normal and abnormal preparations by Nelson and Hitchcock is 0.70 per cent.

described by Nelson and Hitchcock; namely, by boiling until the solution no longer showed any hydrolyzing power, the loss of water by evaporation being corrected for before using.

It is evident from Experiment 22 (Table II) that Preparation 3, which was normalized by the addition of 10 cc. of boiled No. 8 (Nelson and Hitchcock, Experiments 25 and 26), is also completely stabilized by it during the 5 hour incubation period. On the

<sup>2</sup> A. D. has its usual significance in the theory of errors; it represents the average percentage deviation from the mean of the series of  $n$  values for any one hydrolysis.

other hand, with Preparation 6 which was but slightly, if at all, normalized (Nelson and Hitchcock, Experiment 35), there was a corresponding failure of protection against inactivation as shown by Experiments 47 and 48, although they were performed with the enzyme solution after further dialysis. Exactly the same parallelism between the two phenomena occurs with respect to the influence of salt, as becomes evident from a comparison of Experiments 42 and 44 (Table III) with No. 23 (Table II) for Preparation 3, and Experiment 48 (Table III) with Nos. 43 and 44 (Table II) for No. 6.

An observation similar to the above has been reported by von Euler and Laurin (5) who found that a boiled invertase solution could inhibit the inactivation of an unboiled one from the same source. But when the influence of an inactivated bottom yeast extract on a solution from top yeast was investigated, it was found to be nil. With respect to the stabilizing action of sodium chloride on invertase, no reference could be found in the literature, although Willstatter and his coworkers (2) have observed such an effect with calcium chloride. Amylase and ptyalin, however, have been investigated with respect to sodium chloride protection by Ernstrom (6). While salt had a very marked stabilizing action on the latter, so high a concentration as 0.09 M had no appreciable influence on the former. In the case of ptyalin, the effect increases rapidly with concentration of sodium chloride to a maximum at 0.1 M and then falls off slowly. That this distinction between the two enzymes is fundamental is open to question, in view of the fact that different preparations of the same enzyme can, as shown here with invertase, react differently in this respect.

In addition to the normalizing action exerted by sodium chloride and by yeast gum, the variation of degree of abnormality with enzyme concentration was also determined by Nelson and Hitchcock. Examination of the results of their Experiments 58, 12, 54, and 56 reveals the fact that the magnitude of the A. D. decreases with increased concentration of invertase. The values range from 2.7 per cent with 0.5 cc. of enzyme solution per 100 cc. of hydrolyzing mixture, to 0.67 per cent with 6 cc. per 100 cc. In the latter case, the A. D. is actually reduced to a value less than that set for the distinction of normal and abnormal prep-

arations. From the inactivation point of view, this is just the effect that would be predicted. The smaller the concentration

TABLE IV.

Experiment No.	Invertase No.	Inactivation time	Varied condition	$n \times 10^5$	Inactivated
		<i>hrs</i>	<i>pH</i>		<i>per cent</i>
51a	3	0	No Buffer.	855	
b		5	5.8	854	0.0
61a	3	0	5.8	862	
b		5		863	0.0
62a	3	0	5.8	862	
b		5		861	0.0
52a	6B	0	5.8	70.9	
b		5		61.9	12.7
53a	6B	0	5.8	70.8	
b		5		61.7	12.8
54a	7	0	5.8	523	
b		5		522	0.0
3a	8	2	5.8	983	
b		6		983	0.0
59a	7	0	2.4	524	
b		5		448	14.5
60a	7	0	2.4	522	
b		5		447	14.4
35a	8	0	2.4	1,190	
b		5		1,140	4.2
39a	6B	0	2.4	67.3	
b		5		42.2	37.3
40a	6B	0	2.4	67.3	
b		5		42.3	37.4

of enzyme, the greater is the time required for attainment of, say, 90 per cent inversion of the sucrose. But, since the inactivation is progressive, the loss of activity in the course of the

hydrolysis will also increase, and consequently the observed A. D. over these varying periods will grow larger as the dilution of invertase increases.

A comparison of Experiments 70 and 69 with the incubation experiment of Nelson and Hitchcock, referred to in the introduction to this paper, shows that, whereas invertase Preparation 3 loses none of its activity in 5 hours at pH 5.8, at 4.9 it loses 1.1 per cent. Unless the former stability was due to a difference in source of acidity, such as nature of buffer, this difference would indicate that the stability optimum of Preparation 3 is at a more alkaline value than 4.5, the value reported by von Euler and Laurin. Accordingly, after checking the observation of Nelson and Hitchcock (Experiment 51, Table IV), the incubation was repeated at the same pH in the presence of 0.01 M citrate buffer. As can be seen from the results of Experiments 61 and 62, the

TABLE V.  
*Experiments 12' and 12"*  
*Spontaneous Activation of Invertase Hb—pH 3.3*

Experiment No	Inactivation time	$n \times 10^4$ (average).	Activated
	hrs		per cent
a	0	150	0
b	3 5	150	0
c	17	150	0
d	40 5	150	0
e	74	153	2
f	118	151	0 7

added buffer has no appreciable influence. It therefore follows that for Preparation 3 at 25°C., the stability optimum occurs at pH greater than 4.5 and in the neighborhood of 5.8, furnished by citrate buffer.

Regarding the other three enzyme solutions, the results of activations at both pH 6.6 and 2.4 (Table IV) suggest that under these conditions the stability optima of these preparations are also higher than pH 4.5. In the case of No. 6b, the losses at pH 4.9, and 2.4 are 12.8, 2.7, and 37.2 per cent, respectively, the first being about one-third of the last. Similarly, the two normal invertases are stable at both the lower hydrogen ion concentrations while at pH 2.4 the losses are 4.2 and 14.4 per cent or vs. 8 and 7, respectively.

TABLE VI.  
*t* equals time of sampling in minutes.

Experiment 2b.		Experiment 2d.		Experiment 12'e.		Experiment 12'e.	
<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$
25	991	25	990	25 05	1,538	20	1,532
30	988	30	987	35	1,528	30	1,525
35	985	35	987	45	1,530	40	1,524
Mean . .	988	Mean	988	Mean	1,532	Mean.	1,527
Experiment 3a.		Experiment 3b.		Experiment 12'f.		Experiment 12'f.	
<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$
25	979	25	979	25	1,506	20	1,512
30	983	30	985	35	1,506	30	1,507
35	986	35	986	45	1,509	40	1,506
Mean	983	Mean	983	Mean	1,507	Mean	1,508
Experiment 12'a.		Experiment 12'a.		Experiment 18a.		Experiment 18b.	
<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$
19 23	1,509	16	1,513	25	827	25	824
29 1	1,499	26	1,499	30	822	30	819
40 0	1,503	34	1,494	35 1	821	35 1	817
Mean .	1,504	Mean	1,502	Mean	823	Mean	820
Experiment 12'b.		Experiment 12'b.		Experiment 22a.		Experiment 22b.	
<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$
20	1,510	15 17	1,512	39	856	39	857
30 17	1,498	25 4	1,506	55	858	55	858
40 07	1,500	35 33	1,504	70	854	70	852
Mean	1,503	Mean	1,507	Mean	856	Mean .	856
Experiment 12'c.		Experiment 12'c.		Experiment 23a.		Experiment 23b.	
<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$
20	1,504	15 08	1,506	39	791	39	790
30	1,498	25	1,488	55	791	55	794
40	1,497	35	1,488	70	787	70	788
Mean .	1,500	Mean . .	1,494	Mean. . .	790	Mean .	791
Experiment 12'd.		Experiment 12'd.		Experiment 35a.		Experiment 35b.	
<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$
30	1,505	25 25	1,502	15	119	15	114
40 13	1,500	35	1,494	20	118	20	114
50 25	1,501	45	1,497	25	119	25	114
Mean . . .	1,502	Mean . .	1,498	Mean . .	119	Mean . . .	114

TABLE VI—Continued.

Experiment 39a.		Experiment 39b.		Experiment 51a.		Experiment 51b.	
<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$
120	672	120	424	40	858	40	856
150	671	150	422	55	854	55	854
192.3	677	180.2	420	70.1	854	70.1	852
Mean	673	Mean	422	Mean	855	Mean	854
Experiment 40a		Experiment 40b		Experiment 52a		Experiment 52b.	
<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$
120	672	120	424	119.9	715	119.9	623
150	668	150	422	150	707	150	617
168	680	180	424	180	706	180	618
Mean..	673	Mean	423	Mean	709	Mean	619
Experiment 43a		Experiment 43b		Experiment 53a		Experiment 53b	
<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$
119.1	764	120	731	120	716	120	623
150	759	150	725	150	701	150	612
180	750	180	723	180	708	180	616
Mean	758	Mean	726	Mean	708	Mean	617
Experiment 44a		Experiment 44b		Experiment 54a		Experiment 54b	
<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$
120	768	120	729	30	522	30	521
150	761	150	723	35	522	35	523
180	754	180	721	40	524	40	522
Mean	761	Mean	724	Mean	523	Mean	522
Experiment 47a		Experiment 47b		Experiment 59a		Experiment 59b	
<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$
120	767	120	750	30	523	30	446
150	761	150	740	35	524	35	447
180	758	180.1	733	40	526	40	450
Mean	762	Mean	741	Mean	524	Mean	448
Experiment 48a		Experiment 48b		Experiment 60a		Experiment 60b	
<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$	<i>t</i>	$n \times 10^4$
120	765	120	748	30	521	30	444
150	761	150	739	35	521	35	447
180.1	754	180	737	40	523	40	449
Mean	760	Mean	741	Mean.	522	Mean...	447

TABLE VI—*Concluded.*

Experiment 61a.		Experiment 61b.		Experiment 69a.		Experiment 69b.	
<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$
40	863	40	866	41	842	40 5	829
55	863	55	862	55	838	55	833
70	862	70	862	70	839	70	832
Mean	862	Mean	863	Mean	840	Mean	831
Experiment 62a		Experiment 62b		Experiment 70a		Experiment 70b	
<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$
40	861	40	862	40 .	843	40	823
55	864	55	863	55	838	55	831
70	862	70	858	70.5	840	70	830
Mean	862	Mean	861	Mean . . .	840	Mean . .	831
Experiment 63a		Experiment 63b		Experiment 71a		Experiment 71b.	
<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$
40	806	40	791	120	728	120	709
54	801	55	784	150	724	150	706
70	797	70	784	180	729	180	705
Mean	801	Mean	786	Mean .	727	Mean	707
Experiment 64a		Experiment 64b		Experiment 72a		Experiment 72b	
<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$	<i>t</i>	$n \times 10^5$
40	806	40	791	120	728	120	709
55	803	55	787	150	727	150	708
70	798	70	782	180	727	180	705
Mean	802	Mean	787	Mean	727	Mean	707

This is in accord with the observation of Bloomfield (7), that the values for  $n$  decrease during a hydrolysis in the vicinity of pH 2, while at 4.5 and 7, they remain constant. Because of the small amounts of these various preparations available, it has not been possible to examine this point in greater detail.

As a matter of record, the following observations are also reported. In studying the relative stabilities at pH 2.4, invertase No. Hb, a new preparation made as described below, was found to have undergone no inactivation in  $3\frac{1}{2}$  hours (Experi-



ments 12' and 12'', Table V). Continuing the incubations, the activity persisted unchanged for  $40\frac{1}{2}$  hours, but after the 3rd day (74 hours) showed an increase of 2 per cent. This was followed 44 hours later by a drop to 0.7 per cent above the original value. The possibility of this being due to experimental error is eliminated by its magnitude as well as by the duplication of effect. At the same time the 74 hour samples were withdrawn from their respective incubation bottles, it was noticed that a fine white sediment had settled out in each of them.

#### EXPERIMENTAL DETAILS.

*Procedure*—For studying the inactivation, the following solutions were prepared: An incubation mixture consisting of

- (A) 1 Stock invertase solution,
- 2 Buffer solution of such a nature as to give the desired pH, and in such quantity as to yield a concentration of buffer salts equal to 0.01 molar.
- (C) A solution consisting of
  - 1 Sucrose,
  - 2 Buffer combination,
  - 3 Water in which these components were present in such proportions that when a convenient number of cc. of (A) was added to a given volume of (C), the hydrolyzing mixture (C') contains the components in the following concentrations:
    - (a) Sucrose, 10 gm. per 100 cc.,
    - (b) Buffer, 0.01 molar, of pH 4.5,
    - (c) Invertase solution, in such an amount as to give a convenient velocity of hydrolysis.

Throughout the incubation, Solution (A) was kept in the thermostat. In order to follow the progress of the reaction, samples were pipetted out and added to Solution (C) immediately (within 1 minute) after mixing the components and at desired time intervals thereafter. The mean delivery time of the pipette was taken as the time of observation in all cases.

In order to determine the extent of hydrolysis at any time, a 25 cc. portion of (C') was added to 5 cc. of 0.1 M sodium carbonate solution, which stopped the reaction. From  $\frac{1}{2}$  to 2 hours after this, as recommended by Hudson (8), the polariscopic rotations of these solutions were determined. The initial rotation of the hydrolysis mixture was determined by adding a proportionate amount of Solution (A) to the sodium carbonate solution, in order to inactivate the enzyme. Then enough of (C) was added to this so as to make the relative amounts of the three components the same as in any other hydrolysis sample. The final readings were taken 2 or more days after the experiment was performed.

In order to get, as accurate as possible, a determination of the activity of Solution (A), the pH of (C') was always kept at the optimum value, 4.5, thus giving a maximum value to the velocity constant hydrolysis  $n$  for the given invertase concentration. The relative amounts of the buffer components in Solution (C) necessary to give this value of pH, were determined as follows: The amounts of each of these components already present in the portion of Solution (A) added to Solution (C) were subtracted from the corresponding values for the resulting Solution (C') employed in the hydrolysis experiment. When (A) contained more acid than was required in Solution (C') then enough sodium hydroxide was added to neutralize the excess. In an inactivation experiment run over several months, a pH determination repeated after a long interval of time showed no change in the acidity. Such an experiment required the presence of toluene in Solution (A) to avoid growth of mold.

A description of the method of preparation used for all of the invertase solutions with the exception of No. Hb, is given in the paper of Nelson and Hitchcock. The procedure used was essentially that of Nelson and Born (9). In the case of the new preparation, No. Hb, the procedure was practically that of von Euler. The autolyzed bottom yeast juice was precipitated with an equal volume of 95 per cent alcohol, the gum taken up with distilled water and reprecipitated. An aqueous solution of the gum so obtained was used without being dialyzed.

The pH was determined colorimetrically, using the necessary Clark and Lubs indicators (10). The citrate standards used were calibrated electrometrically with bubbling hydrogen electrode against a saturated potassium chloride calomel cell, joined by a saturated potassium chloride bridge. The calculations were based on 0.1 *N* hydrochloric acid as a standard, its conductance dissociation being taken as 92.04 per cent at 25°C., at which temperature all determinations were made.

The light source used in the polariscopic work was a mercury vapor arc.

#### SUMMARY.

1. An explanation based on their lower relative stability has been offered for the "abnormality" of certain invertase preparations observed by Nelson and Hitchcock and by Willstätter and his coworkers.

2. It has been shown that sodium chloride and yeast gum exert a stabilizing influence on one, but not on the other, of the two abnormal preparations studied.

3. Data were obtained at 25°C. showing that the stability optimum of one abnormal invertase preparation did not occur in the pH region of optimum activity as suggested by von Euler and Laurin for the temperature interval 45–55°, but further towards the alkaline side; *i.e.*, in the vicinity of pH 5.8.

4. Likewise, data obtained with the two normal preparations suggest that they also do not agree with von Euler and Laurin's conclusion in this respect.

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## COLLODION MEMBRANES OF HIGH PERMEABILITY.\*

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Various methods of preparing collodion membranes of graded permeability have been previously described by Bechhold (1), Walpole (2), Brown (3), and Eggerth (4). These several investigators have arrived empirically at different procedures for producing essentially the same result. When, however, a reproducible graded membrane of high degree of permeability is desired, none of their methods is entirely satisfactory.

In the work described in this paper, flat membranes, prepared according to Walpole, were used. By Walpole's procedure collodion solution is poured upon a carefully levelled circular glass plate in sufficient quantity to spread evenly to the edges of the plate. As the alcohol-ether solvent evaporates the solution becomes more and more viscous until finally a membrane is formed. The plate, with its film, is now immersed in water and after the lapse of a few minutes the membrane can be easily removed.

Walpole observed that by water immersion of the plate and film at different stages in the evaporation of the solvent, a series of membranes of different degrees of permeability could be obtained, since in this way the amount of residual solvent to be replaced more or less by water is varied. This relationship between the amount of water and solvent finally contained in the film, which Walpole has termed the "wetness," and the degree of permeability of the film has since been substantiated by Brown (3) and Eggerth (4).

In the present work the "wetness" per gram of dry collodion is termed the "grade" of the membrane and is determined directly

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by weighing. This procedure constitutes an improvement over Walpole's measurement of the thickness, since it permits determination of the grade of a membrane at any point in the process without waiting until the membrane becomes tough enough to measure with a caliper. A further advantage is the possibility of working with very much thinner membranes (about 0.01 mm. versus Walpole's 0.1 mm.).

In working with these thin membranes the very interesting observation was made that the change from viscous liquid to rigid jelly is quite abrupt. It thus becomes possible to detect and measure an important property of the membrane; namely, the grade of the membrane at the instant of jellification, the so called "initial gel grade."

By means of this initial gel grade it is possible to measure the tendency of a given sol to set to a jelly, and a direct way is thereby provided for studying the conditions favorable to the formation of jellies of high degree of wetness and hence of high permeability. From the foregoing it is obvious that one very important condition favoring high permeability is the immersion of the membrane in water as quickly as possible after the point of jellification has been reached.

### *Relation between the Composition of the Collodion Sol and the Initial Gel Grade.*

The actual method finally adopted for this work combines several features of the different techniques previously mentioned. Thus the solutions are prepared, as by Eggerth (4), by dissolving a given weight of Du Pont's parlodion in 100 gm. of absolute alcohol and ether, mixed in stated proportions by weight; and a measured volume of the given solution is then poured, as by Walpole, on a plate of known weight. If too much collodion is used the surface of the thick membrane formed gels somewhat sooner than the main portion of the liquid and there is consequently no *sharp* point of transition from sol to gel. With a sufficiently thin membrane, however, this transition is abrupt and is readily observed. If changes in the consistency of the drying film are followed by gently touching it from moment to moment with a pointed match it will be observed that somewhat before jellification the rapidly thickening material adheres

strongly to the match as the latter is withdrawn; then, in quick succession, pressure with the match produces a slight temporary depression of the gelled surface into the still liquid sublayer, and, *immediately*, afterward, a semipermanent deformation, indicative of complete rigidity or jellification throughout the entire film. At this instant the plate is placed on the balance and weighed as quickly as possible to the nearest 10 mg. This weighing gives  $W_o$ , the weight of collodion plus the organic solvent retained in the jelly. As the solvent is evaporating at the rate of about 50 mg. a minute and as it takes about half a minute to make the weighing the measurements will run low by about 25 mg. However, since this error is always in the same direction and since the weights obtained in different experiments are found to differ by hundreds of milligrams no correction was introduced for this error.

As soon as the weight is determined the plate is immersed in water and left there 15 or 20 minutes until, on raising it, the water is found to wet the membrane surface. The membrane can now be weighed, after drying between filter papers, either on the plate, or by itself after removal therefrom. This determination,  $W_w$ , is more accurate than that of  $W_o$ , as the water evaporates at the slower rate of about 10 mg. per minute from the membranes used. The dry weight,  $D$ , of collodion is finally determined to about 0.5 mg. after drying overnight at 70°C. From these data,  $G_o$ , the grams of solvent per gram of collodion before water immersion, and,  $G_w$ , the grams of solvent per gram of collodion after water immersion are calculated as follows:

$$G_o = \frac{W_o - D}{D}$$

$$G_w = \frac{W_w - D}{D}$$

$G_o$  is the "grade" of the organo-jelly, and  $G_w$  is the "grade" of the final membrane.

Using the above described procedure the first question investigated was the effect on the initial gel grade of variation in the parlodion content of solutions of constant alcohol-ether composition. Previous work by Bechhold (1) indicated clearly

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the existence of such an effect. In each experiment 50:50 (by weight) alcohol-ether solutions of suitable parlodion content were used. Each membrane was numbered by inserting a small paper tag in the rim of the jelly. The results are shown in Table I.

TABLE I

*Effect of Variations in the Parlodion Content of the Sol upon the Initial Gel Grade,  $G_o$ , and the Final Grade of the Membrane,  $G_w$ .*

Alcohol-ether ratio = 50:50 in each experiment.

Mem- brane No	Parlo- dion per 100 gm solvent	Sol used	Time to set	$W_o$	$W_w$	$D$	$G_o$	$G_w$	$R$
	gm	cc	min						
108	6	10	13	3 413	2 068	0 423	7 1	3 9	1 82
109	6	10	9	3 382	1 965	0 423	7 0	3 6	1 94
110	4	8 5	10	2 892	1 830	0 247	10 7	6 4	1 67
111	4	8 5	9	2 790	1 760	0 247	10 3	6 1	1 69
112	4	8 5	95	2 875	1 850	0 245	10 7	6 1	1 76
113	4	8 5	95	2 832	1 760	0 243	10 7	6 2	1 73
114	3	7 5	9	2 500	1 725	0 163	14 3	9 6	1 49
115	3	7 5	85	2 413	1 650	0 164	13 7	9 1	1 51
116	3	7 5	9	2 542	1 755	0 166	14 3	9 6	1 49
117	3	7 5	9	2 440	1 630	0 166	13 7	8 8	1 56
118	2	5	6	1 404	0 960	0 086	15 3	10 2	1 50
119	2	5	6	1 645	1 150	0 092	16 9	11 5	1 47
120	2	5	6	1 500	1 084	0 086	16 5	11 6	1 42
121	2	5	6	1 450	1 070	0 086	15 9	11 4	1 40
122	2	5	6	1 415	1 050	0 086	15 4	11 2	1 38
123	2	5	6	1 483	1 085	0 089	15 7	11 2	1 40

The first four columns give, successively, the number of the membrane, the grams of parlodion per 100 gm. of solvent, the volume in cubic centimeters of sol required to cover the plate, and the time required for the jelly to form. The next three columns give the observed weights of the membranes:  $W_o$ , the combined weight of parlodion and organic solvent at the moment the jelly sets;  $W_w$ , the weight of parlodion plus solvent after immersion; and  $D$ , the weight of the membrane after drying overnight at 70°C. From these data,  $G_o$ , the "initial gel grade,"

that is, the grams of organic solvent per gram of parlodion before immersion; and  $G_w$ , the grams of solvent per gram of parlodion after immersion, is calculated as previously described.

In the last column, headed  $R$ , is given the ratio of the grade before and after immersion in water.

$$\left( R = \frac{W_o - D}{W_w - D} \right)$$

A comparison of the values of  $G_o$ , the initial gel grade, for groups of membranes made from solutions of the same collodion content shows that the new method of determining this property gives satisfactory results. The reliability of the method seems therefore established. A further point of interest is brought out; namely, that the initial gel grade,  $G_o$ , from a 2 per cent sol is more than twice as large as  $G_o$  from a 6 per cent sol.

This would naturally suggest that further increase in grade could be attained by the use of still more dilute solutions, but experiment quickly demonstrated that the resulting films are too fragile to be used. The remaining experiments in this paper have therefore been carried out with 2 per cent solutions.

Having established an optimum parlodion concentration we next studied the effect of varying the alcohol-ether ratio in the solvent mixture. The existence of such an effect was indicated clearly by the work of Eggerth (4). The application of the initial gel grade method to this problem yielded the results shown in Table II.

The results in the  $G_o$  column agree in general with the work of Eggerth in showing that the grade increases with alcohol content of the solvent. Not only, however, has the present method yielded membranes of a grade more than twice as high as any reported by Eggerth (compare our  $G_w = 17$  with Eggerth's highest grade of  $G_w = 8.43$ ); even more to be emphasized is the elimination by the present technique of the uncertainty involved in the employment of any drainage method. When films are produced by dipping and drainage, as in Eggerth's work, the properties of the membranes produced depend upon the simultaneous influence of at least three factors: (1) the viscosity of the solution which affects the rate of drainage and consequently the thickness of the film at various levels; (2) the rate of evaporation,



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which affects, on the one hand, the drainage, and, on the other, the rate of gel formation; and (3) the critical concentration at which jellification occurs. The initial gel grade method eliminates drainage as a factor and likewise by referring all observations to a standard condition of the film, regardless of the time consumed in reaching that condition, eliminates rate of evaporation, leaving the critical concentration as the only variable. The favorable effect of increased alcohol concentration on the grade of the membrane can therefore now be definitely attributed to an influence on the sol-gel transformation.

TABLE II

*Effects of Variations in the Ratio of Alcohol to Ether in the Solvent.*  
5 cc. of 2 per cent parlodion sol were used in each experiment.

Alcohol- ether ratio by weight	Membrane No	$W_o$	$W_w$	$D$	$G_o$	$G_w$	$R$
25:75	124	0 904	0 665	0 083	9 9	7 0	1 42
	125	0 876	0 640	0 085	9 3	6 5	1 43
	126	0 980	0 690	0 084	10 6	7 2	1 47
	127	0 933	0 645	0 085	10 0	6 6	1 52
50:50	118	1 404	0 960	0 086	15 3	10 2	1 50
	119	1 645	1 150	0 092	16 9	11 5	1 47
	120	1 500	1 084	0 086	16 5	11 6	1 42
	121	1 450	1 070	0 086	15 9	11 4	1 40
75:25	128	1 912	*	0 081†	21 9		
	129	2 101	1 480*	0 082	24.6	17 1	1 44
	130	2 082	1 410*	0 079	25 4	16 9	1 5
	131	2 045	1 380*	0 082	23 9	15 8	1 51

\* Damaged in removal from plate.

† 0 081 is the average  $D$  for Membranes 129, 130, and 131

The practical employment of our most permeable membranes in a study of the diffusibility of invertase was prevented, however, by the impossibility of removing them intact from the plate. Fortunately, a very simple way of overcoming this difficulty was soon found, for it is only necessary to fog the glass surface before pouring on the collodion sol in order to obviate the tendency to stick. This is readily accomplished by breathing on the plate which, if necessary, has been chilled to a few degrees below room

temperature. A light fog is all that is necessary; a heavy deposit of moisture is to be avoided, as the membrane may be damaged. Fogging also provides a test for the cleanness of the surface, for, if an invisible film of collodion or oil is present, the fog will not form evenly.

It is of interest to note that even the small amounts of moisture introduced into the membrane by this fogging process have a well defined effect on the initial gel grade. To illustrate this point Table III is introduced. In each experiment of this series 5 cc.

TABLE III  
*Effect of Fogging the Plates.*

5 cc. of 2 per cent parlodion sol of alcohol-ether ratio 75:25 were used in each experiment

	Membrane No	$W_o$	$W_w$	$D$	$G_o$	$G_w$	$R$
Plates not fogged	128*	1 912		0 081†	21 9		
	129*	2 101	1 480	0 082	24 6	17 1	1 44
	130*	2 082	1 410	0 079	25 4	16 9	1 5
	131*	2 045	1 380	0 082	23 9	15 8	1 51
Plates fogged	136‡	2 273	1 565	0 079	27 8	18 8	1 48
	137‡	2 286	1 555	0 080	27 6	18 5	1 49
	138‡	2 282	1 580	0 081	27 2	18 5	1 47
	139‡	2 264	1 540	0 080	27 3	18 3	1 49
Plate not fogged, 2 per cent H <sub>2</sub> O in sol	146*	2 602	2 086	0 080	31 5	25 1	1 26
	147*	2 534	2 000	0 080	30 7	24 0	1 28
	148*	2 612		0 080†	31 6		
	149*	2 546		0 080†	30 9		

\* Damaged in removal from plate

† Average  $D$  for Membranes 129, 130, and 131.

‡ Came off plate easily

of a 2 per cent parlodion, 75:25 (by weight) alcohol-ether sol, were used. The membranes were immersed in water as soon as the initial gel weight was determined. The effect of moisture on the initial gel grade,  $G_o$ , is evident from comparison of Experiments 128 to 131, where no moisture was deliberately added, with Nos. 136 to 139, where moisture was added by fogging the plate, and with Nos. 146 to 149, where moisture was added directly to the solution.

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These results suggested a new way of increasing the grade of the membranes, but unfortunately, very little advantage could be gained by adding water to the sols because even small amounts of water have the undesired effect of making the membranes too fragile for use. The practical value of these observations consists in the parallel increase in initial gel grade and fragility, thus suggesting precautions to be adopted in order to obtain films of high grade, and at the same time reasonable strength. Aside from the obvious precautions taken for the dryness of the solvent alcohol-ether, there are still two ways by which moisture may get into the membrane. First, the worker may inadvertently breathe on the solution on the plate while watching for the initial gel

TABLE IV

*Effect of Humidity on the Initial Gel Grade,  $G_o$ .*

In each experiment the plate was fogged and 5 cc of a 2 per cent parcollodion sol of alcohol-ether ratio (by weight) equal to 75/25 were poured upon it

	Membrane No	$W_o$	$D$	$G_o$
Relative humidity less than 50 per cent.	171	1 904	0 076	24
	172	1 934	0 076	24 4
Relative humidity, sol breathed on.	173	2 346	0 076	29 9
	174	2 278	0 076	29 0
Relative humidity about 80 per cent.	176	2 213	0 076	28 1
	177	2 338	0 076	29 7
	178	2 342	0 076	29 8
	179	2 309	0 076	29 4

point; and second, if the relative humidity be sufficiently high, moisture may be taken up from the atmosphere to the detriment of the membrane. Cases illustrating these effects are shown in Table IV, which is self-explanatory.

We suggest as a plausible, though incomplete, explanation of the rôle of water in these experiments that moisture in the sol causes a precipitation of the collodion concomitantly with gel formation. This tentative explanation seems justified by the observation that the fragile jellies are more opalescent than the stronger ones and that relatively slight further increases in water content cause distinct precipitation in separate white flocks.

It will be noted from our tables that the ratio  $G_o:G_w$  is always greater than unity. This indicates a loss of permeability during the immersion of the membrane in water. It was thought that if the replacement of organic solvent by water were allowed to take place more gradually these losses might be diminished. An increase in the grade after water immersion was actually accomplished by dipping successively in alcohol-water solutions of decreasing alcohol strength. The results are shown in Table V.

TABLE V.

*Effect on Final Grade,  $G_w$ , of Immersing the Organo-Gel in Alcohol-Water Solutions of Successively Lower Alcohol Strengths.*

5 cc. of 2 per cent parlodion sol of alcohol-ether ratio = 75:25 were used in each experiment

	Mem- brane No	$W_o$	$W_w$	$D$	$G_o$	$G_w$	$R$
Plate fogged; direct water immersion.	136	2 273	1 565	0 079	27 8	18 8	1 48
	137	2 286	1 555	0 080	27 6	18 5	1 49
	138	2 282	1 580	0 081	27 2	18 5	1 47
	139	2 264	1 540	0 080	27 3	18 3	1 49
Plate fogged; graded alcohol immersion	161	2 210	1 800	0 076*	28 1	22 7	1 24
	162	2 150	1 730	0 076	27 3	21 8	1 25
	164	2 230	1 840	0 076	28 3	23 2	1 22
	165	2 186	1 875	0 076	27 8	23 6	1 13

\* 0 076 is the average  $D$  for three other membranes prepared in the same way

In the last five experiments (Nos. 161 to 165), the membranes were immersed successively in alcohol-water solutions of the following strengths, by volume, of 95 per cent alcohol: 80, 70, 60, 50, 40, 30, 20, 10, and 0 per cent. A membrane is kept in each succeeding solution from 2 to 10 minutes until on raising it the surface is wetted by the solution.

It is to be noted first, that the initial gel grade,  $G_o$ , values agree very well throughout both groups of experiments and second, that the final grade,  $G_w$ , shows a considerable increase from an average value of about 18.5 (in the first series) to an average value, in the graded alcohol experiments, of about 23.1. The column headed  $R$  shows the marked change in  $G_o:G_w$  from the usual value of about 1.5 to a lower value of about 1.2. These mem-

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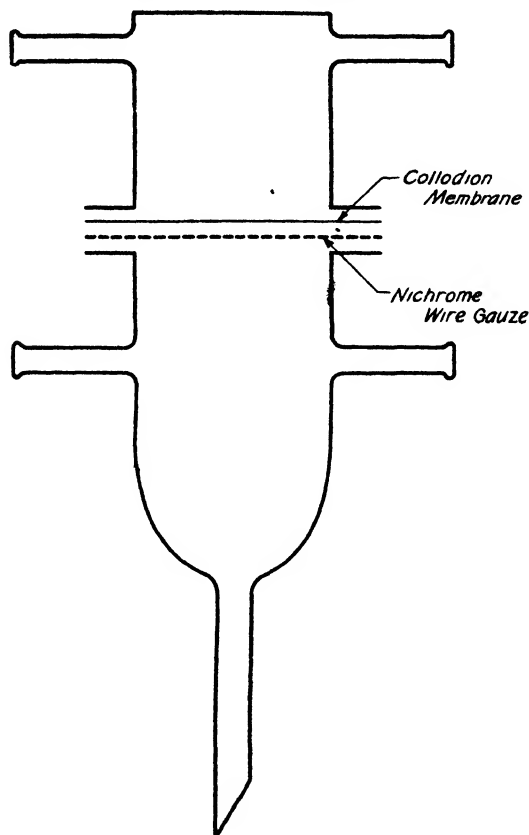
branes, and others of the same grade, were easily removed from their plates and were actually used in experiments on the diffusibility of invertase.

An additional point of practical importance is the possibility of preparing by our method in a simple manner a membrane of any desired permeability, by allowing one of a higher grade to evaporate on the balance till the desired weight is reached. Thus to make a series of membranes of  $G_w$  equal to 23 it is necessary only to make a number of membranes whose  $G_w$  is higher than this and evaporate them down to the desired grade.

A final difficulty which had to be overcome in order to render our membrane suitable for the study of invertase arises from the unavoidable variations in permeability at different parts of the film. This phenomenon was observed by Bechhold (5) when he forced air under known pressure through the membrane into a thin layer of supernatant water. Certain points in the surface allow the passage of air at pressures insufficient to force air generally through the film. The precaution was therefore taken of using a mat of three membranes in each experiment reported below with the hope of reducing the chance of one part of the surface being very much more permeable than another.

*Procedure for Membrane Preparation.*—As a result of the previous experiments, the following procedure for preparing membranes for ordinary use has been developed. The outside diameter of the flanged tubes of the filtration apparatus to be described later was 3 inches, and, therefore, 4 inch circular glass plates were used for the preparation of the membranes. One of these plates (the plates were always cleaned before using with cleaning mixture—concentrated sulfuric acid, saturated with potassium dichromate—washed, rinsed with dust-free distilled water, and dried in the oven at 50°C.), chilled if necessary, is weighed, carefully levelled on the desk, fogged, and 5 cc. of a 2 per cent solution of Du Pont's parlodion in 75:25 (by weight) alcohol-ether solvent mixture are run upon it from an ordinary pipette and allowed to evaporate. It is a good idea to guard the solution, as it evaporates, from convection currents and draughts by a cylindrical shield which also slows the process, making evaporation more even, and decreases the chance of moisture from the atmosphere spoiling the membrane. As soon as a jelly

forms, the plate is removed to a balance and evaporation is allowed to proceed down to a weight which has been found by a trial or two to give a grade after water immersion ( $G_w$ ) somewhat higher than that desired. The immersion may be in water directly, or



### *FILTRATION APPARATUS*

FIG. 1.

this may preferably be preceded by immersion in graded alcohol-water mixtures. The water-wet membrane is worked loose from the plate under water by gentle lateral pressure of the finger tips, and set aside to soak in water overnight. This soaking is advised because it is occasionally found that a membrane which has

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possibly been hurried through the solvent replacement process will lose weight during the first few hours. After this alteration has taken place, the membranes will keep their grades for weeks. At the same time that the soaking process is taking place, a few membranes are dried in the oven to get the dry weight ( $D$ ), which must be known to calculate the  $W_w$  corresponding to a certain desired  $G_w$  or *vice versa*. When a series of membranes of variable grades but all greater than the one desired is ready, it is only necessary to evaporate each on the balance to the predetermined weight  $W_w$ . The only important error in  $G_w$  is in the assumption that all membranes made from the same solution will have the same dry weight, but on the average this error is found to be less than 2 per cent. For example, the dry weights ( $D$ ) of four samples from one series of membranes were in grams, 0.077, 0.076, 0.080, and 0.077, where the average deviation from the mean is 1.3 per cent.

*Apparatus.*—For the convenient use of these membranes in ordinary work a special apparatus was necessary. In Fig. 1, one form of this apparatus is shown which was designed and used in filtration experiments. Except for the differences in the inlet and outlet tubes the two parts are essentially the same. They consist simply of two pieces of heavy walled tubing 2 inches in diameter which are flanged and ground to fit each other. The convenient feature is the method of holding the two flanges tightly together when the membrane is placed between. This is accomplished very simply and effectively by snapping a number of strong rubber bands over the arms.

In experiments where it was necessary to brace the membranes this was satisfactorily accomplished by the use of an ordinary nichrome gauze. Such a gauze is sufficiently stiff, yet has a comparatively open mesh. In order to make this system water- and air-tight the meshes of the gauze where they come in contact with the flanges, are filled with a special gummy preparation consisting of rubber dispersed in paraffin. (This and several other valuable suggestions were given to the authors by Dr. T. C. Taylor of this University.)

In the filtration experiments about to be described this apparatus was set up in a suction flask which was connected to a mercury or water manometer and an ordinary suction pump.

This relatively simple arrangement is possible because only low pressures are needed due to the great permeability of the membranes and the low osmotic pressures developed by the colloidal solutions investigated. (Compare Bechhold (1) and McBain and Jenkins (6).)

TABLE VI.

*Filtration Experiment F<sub>3</sub>-V-A, Using Invertase Solution "W. E. M." at pH 7.5, External Pressure (about 0.03 Atmosphere) and Time (11 Hours)*  
Same as in Experiment F<sub>3</sub>-V-B.

Membranes  $\left\{ \begin{array}{l} 156 (G_w = 21.8) \\ 158 (G_w = 22.5) \\ 159 (G_w = 22.1) \end{array} \right\}$

	$V_{O,R,F}$	$K_{O,R,F}$	$K_x = K_o$ reduced to $V_R$ or $V_F$	Distribu- tion of invertase.
	cc.			per cent
Original invertase solution, <i>O</i>	60	0.00330		
Residual solution, <i>R</i>	22	0.00383	0.0090	42.6
Filtrate, <i>F</i> . . . . .	36	0.00243	0.0055	44.2
Total accounted for				86.8
Lost (bv difference), per cent				13.2

TABLE VII

*Filtration Experiment F<sub>3</sub>-V-B, Using Invertase Solution "W. E. M." at pH 7.5, External Pressure (about 0.03 Atmosphere) and Time (11 Hours)*  
Same as in Experiment F<sub>3</sub>-V-A

Membranes  $\left\{ \begin{array}{l} 163 (G_w = 23.2) \\ 164 (G_w = 23.6) \\ 165 (G_w = 23.3) \end{array} \right\}$

	$V_{O,R,F}$	$K_{O,R,F}$	$K_x = K_o$ reduced to $V_R$ or $V_F$	Distribu- tion of invertase
	cc			per cent
Original invertase solution, <i>O</i>	60	0.0033		
Residual solution, <i>R</i>	19	0.00427	0.0104	41
Filtrate, <i>F</i> . . . . .	40	0.0022	0.00495	44.7
Total accounted for . . . . .				85.7
Lost (by difference) . . . . .				14.3



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### *Filtration Experiments.*

The high degree of permeability and the reproducibility necessary for a study of the diffusibility of invertase seemed now to be attained, and, therefore, we proceeded to test our membranes by actual filtration experiments. Equal volumes of the same invertase solution were placed in two of the above described vessels and subjected to ultrafiltration through equal areas of filter for equal times under identical external pressures of about 3 cc. of mercury.

The concentrations of invertase in the original solution, in the residual solution, and in the filtrate were assumed proportional to the monomolecular constant ( $K$ ) of inversion of cane-sugar when equal volumes (2.0 cc.) were added to 98 cc. of 16 per cent sugar solution, buffered by citrate at pH 4.5. Comparison of the observed  $K$  with the  $K$  calculated on the assumption that all the invertase has been concentrated in the observed volume of filtrate or residual solution gives a measure of the yield of sucrolytic material present. The results of two such experiments are shown in Tables VI and VII.

The data show that the membranes are quite permeable to invertase and that two different set-ups can be made which check fairly well. The fact that the residual solutions were dark brown, while the filtrates which contained a considerable portion of the invertase were nearly water-white, points to the possibility of a purification of invertase solutions by fractional ultrafiltration, and it is hoped that the optimum conditions for the application of this method of purification can be reported in a succeeding paper.

### SUMMARY.

1. A method has been devised for studying conditions requisite to the formation of collodion membranes of high permeability.
2. By means of this method the optimal concentrations of collodion and alcohol for ordinary working solutions have been roughly defined.
3. By means of an improved technique membranes have been prepared, characterized by reasonable strength, and, at the same time, a permeability almost three times as great as any previously reported.

4. A simple procedure is outlined for the preparation of a series of membranes of any desired grade, provided only that the grade desired is below the limit fixed by the fragility of the membranes.

5. Under obvious precautions the suitability of such membranes for the study of invertase diffusibility has been established.

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## AQUEOUS EXTRACTS OF PANCREAS.

### II. PHYSICAL AND CHEMICAL BEHAVIOR OF INSULIN.

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Much has yet to be learned regarding the physiology of insulin: how it is formed, how it affects the combustion of glucose, what stimulates its secretion into the blood stream and lymph, and so on. Knowledge in these directions will advance more rapidly as its physical and chemical properties become better known. Progress toward its chemical isolation and synthetic production may likewise be accelerated by disclosures, as rapidly as they are made, of its physical and chemical modes of behavior. The following observations have been made in the course of the past 2 years. Some of them have been reported in preliminary communications (1, 2), but most of them are reported here with full supporting data for the first time.

Insulin is not a protein. In the purest form in which it has been obtained in this laboratory and in concentration sufficient to give a marked hypoglycemic effect with normal rabbits it gives none of the usual protein reactions. In this statement we are in agreement with Best and Macleod (3) as regards their preparation from the pancreas of the skate. It should be noted, however, that our statement applies to the product obtained from the pancreas of the ox and pig. Doisy, Somogyi, and Shaffer (4) state that the substance isolated by them appears to be an albumose or a globulin since it is thrown down by half saturation with ammonium sulfate and when separated by an isoelectric precipitation it gives a distinct biuret reaction, faint reaction with glyoxylic acid, and doubtful Millon's. Best and Macleod by their "usual method" (presumably the original Collip method (5)) had not been able to produce an extract from the pancreas of the ox or pig which was

free of protein. That the protein is an impurity they infer from the fact that after precipitation with phosphotungstic acid and removal of the latter with ether and barium hydroxide the preparation retained its power to cause hypoglycemia. Details of the method of preparing a protein-free extract of ox or pig pancreas which has been evolved in this laboratory will be found in a subsequent paper together with further discussion of its chemical composition.

*Physical Properties.*

1. *Solubility.*—Insulin has been obtained by us as a white or greyish white amorphous powder. In the living pancreas it seems to exist in such a combination with protein that it requires special means to effect its separation. The combination is readily soluble in acidulated alcohol, as the Toronto and St. Louis laboratories have fully demonstrated, and in acidulated water, as this laboratory has shown. Judging by Collip's method which employs alcohol alone, the acid is not absolutely essential to render the protein-insulin complex soluble, and in the perfusion work (6) which has been carried out in this laboratory weak alkali as well as weak acid sufficed to extract the complex. Distilled water which has been tried only with pig pancreas failed to remove it from the cells. It does not seem possible that the destructive action of trypsin is responsible for this failure, for both Clark (7) and our own group obtained insulin under conditions which were favorable to the action of trypsin if trypsin had been present. Several attempts to demonstrate its presence in perfusates failed. It is not impossible that either weak alkali or weak acid produces a soluble metaprotein with which the insulin comes out in close association if not in actual combination. Precipitation of the acid metaprotein by neutralizing the acid partially liberates the insulin, the exact end-point depending upon the amount of protein present (8).

We have had but little experience with alcoholic extraction, hence we are not qualified to speak of the effects of different quantities of acid in this medium; but in aqueous medium we have nearly always obtained distinctly more potent extracts with 0.2 N HCl than with any other strength, and this applies as well to the percolation method which will be described elsewhere (8). The best volume relations for ordinary extraction whether with

or without heat have been 4 volumes of the extracting liquid to unit weight of macerated pancreas. Associated with metaprotein as it is in these aqueous extracts or percolates the insulin then is definitely soluble in 0.2 N HCl.

The volume relation which gives the best result depends upon the amount of acid bound up and thus rendered inactive for the destruction of trypsin. In one extract (No. 58A) which was made by using only 1 volume of 0.2 N HCl so much of the acid was tied up in this way that trypsin was still very active and caused the death of a dog when it was injected subcutaneously. The autopsy finding was not unlike that already described for Dog 2 in our first paper<sup>1</sup> in this series. Many other attempts to reduce the volume of acidulated water have failed and for the same reason. On the other hand, larger volume than four times the mass of macerated tissue and greater strength than 0.2 N HCl have not improved the yield.

These observations on solubility apply to the first stage extraction, a process which is by no means dependent upon simple solubility. As will be described elsewhere (8) the movement of the solvent, the temperature, and automatic filtration of the liquid are important factors.

Further observations on solubility have been made on the finished product as it was prepared for many months for use with diabetic patients. This method which has not been described hitherto may be stated briefly at this point. It has some features in common with the procedure of Zuelzer (10) and the original method of Collip (5).

*Early Method of Purifying Insulin*—Pancreas of beef, taken directly from the slaughtered animals and trimmed hastily of extraneous tissue, was cut into small pieces and dropped at once into 0.2 N HCl, chilled to near the freezing point. It was brought to the laboratory in this condition, passed through a meat grinder, and distributed among the three jars of a bacteria grinder of the Army Medical School type. Fresh 0.2 N HCl was added in proportion of 4 volumes to a unit weight of pancreas. The grinder was usually run overnight (12 to 18 hours). The next morning the finely ground "mash" was strained through cheese-cloth and squeezed as dry as possible by hand. The turbid fluid, containing many small particles of tissue and much protein in solution, was rapidly neutralized with stirring

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<sup>1</sup> Murlin, Clough, Gibbs, and Stokes (9), p. 269.

with  $N$  NaOH to pH of about 6.0. A heavy precipitate of the acid meta-proteins was obtained, and was removed by filtration through plaited filter paper.

Alcohol, 95 per cent, was added to the filtrate in amount equal to  $\frac{1}{2}$  to 1 volume and the mixture placed in vacuum stills at an outside temperature of 34 to 40°C. Distillation continued, usually overnight, or, until the volume had been reduced to one-tenth the original. The acidity was adjusted to a pH of approximately 6.0 and the resulting precipitate filtered off. The filtrate was dialyzed in thin parchment bags for 4 hours in running tap water to remove the salt resulting from neutralization. Any fat extracted was congealed by the cold tap water and was filtered off at this point. The clear filtrate was then treated with 4 volumes of 95 per cent alcohol for further precipitation of proteins. After standing 1 day the product was filtered and concentrated once more in the vacuum still to one-fortieth the original volume after the first neutralization.

The product obtained in this way, like the product obtained by Best and Macleod and by Shaffer and his colleagues, contained usually a trace of protein as shown by the biuret reaction. Sterility was insured partly by the last treatment with alcohol and in part by aseptic filtration into sterile vials. Tricresol was added to 0.1 to 0.2 per cent. This extract was given to diabetic patients (11) in Rochester Hospitals with very satisfactory results.

By this method it was possible to recover in the final product from 100 to 200 rabbit units<sup>2</sup> per 1,000 gm. of pancreas used. Extraction of the residues and precipitates with 80 per cent alcohol would sometimes add some 30 to 50 rabbit units to the yield. This total yield has been greatly increased by recent improvements (8) in the method of extraction.

The spontaneous precipitation of insulin from the final product was witnessed a number of times before Doisy, Somogyi, and Shaffer reported upon the isoelectric point, but no exact measurements of the H ion concentration had been made. Since that time we have confirmed the location of the isoelectric point *in the product containing a small amount of protein* at a range of pH 4.3 to 5.7 (electrometric). We dare not infer, however, that this is the isoelectric point of insulin itself. The solubility of the

<sup>2</sup> More than three times the clinical unit of the Toronto Laboratories. For a full discussion of the rabbit unit adopted in this laboratory see the paper by Clough, Allen, and Root (Clough, H. D., Allen, R. S., and Root, E. W., Jr., *Am J. Physiol.*, 1923, lxxvi, 461 on A study of the rabbit as a test animal for determining the potency of insulin preparations.

purest product we have obtained will be discussed in a subsequent paper. For the present it is sufficient to say that if one wishes to obtain a large yield of insulin by the method described above or by the methods described recently by the Connaught laboratories (12) or by Eli Lilly and Company (13) it is necessary to avoid the range of acidity between pH 4.3 and 5.7 at any stage just preceding a filtration. These figures apply to beef pancreas under the conditions described.

TABLE I.  
*Effect on Potency of Heating to 80°C. for 30 Minutes.*

Preparation No	Stage	Original test Drop in gm per 100 cc	Reaction at time of heat- ing	Subse- quent test Drop in gm per 100 cc	Remarks.
108 x	Final ex- tract	0 068*	pH 7 2	0 091	
108 y	" "	0 031	7 2	0 069	
122 (I)	First fil- trate.	0 061	5 7	0 00	Precipitate fil- tered off.
122 (II)	" "	0 021	5 0	0 00	" "
		0 037			
122 (III)	" "	0 00	4 2	0 00	" "
		0 039			
123	" "	0 071 (3 hrs )	4 3	0 023	" "
		0 075 (3 " )		0 024	Potency re- covered.
123	First fil- trate	0 042 (3 " )	4 0	0 078	Turbid, not filtered.
123 A	" "	0 042	7 2	0 064	Precipitate fil- tered off.
				0 025	
123 B	" "	0 042	6 2	0 047	" "
				0 067	

\*Except where otherwise noted time is 2 hours.

2. *Thermostability.*—The first evidence of the thermostability of the internal secretion of the pancreas is found in a publication by DeMeyer (14) in which he describes the effect of extracts of heated pancreas upon the formation of glycogen. This was followed by the observation of Waterman (15) in 1913 that the active substance contained in the blood of the pancreatic vein which diminished the disappearance of glycogen from the liver in



chloroform narcosis, was thermostable. Murlin and Kramer (16) the same year prepared a pancreatic extract in 0.2 N HCl which was *brought to boiling* temperature, then neutralized with sodium carbonate, and filtered. Injected into the vein of a depancreatized dog it stopped the excretion of sugar in a little over 2 hours. Exactly the same process has been carried out in this laboratory a number of times within the past 2 years with marked effect (9), not only on the excretion of sugar but on the hyperglycemia as well. The reason for some earlier failures by this method, in all probability, was that the insulin was precipitated at the isoelectric point in the process of neutralizing. The range of reaction within which it is safe to heat the final water-soluble product to 80°C. for  $\frac{1}{2}$  hour is shown in Table I. Tests are given on different fractions of three different extractions. The first (No. 108) represents the final product prepared by a method essentially like that described on page 323. The two fractions differ only in the length of time they were subjected to dialysis. Brought to a reaction slightly on the alkaline side of neutrality and heated, there was in both cases an apparent increase in potency. Preparation 122 was tested in the first filtrate stage which contains a considerable amount of protein. Upon heating each fraction showed a marked precipitate which was filtered off before making the subsequent test. Since the reactions fell within the range of the isoelectric precipitation the loss of potency is not due to heat but to the precipitation, hastened no doubt by the coagulation of proteins. Preparation 123 tested within this range of reaction likewise suffered a loss of potency and from the same cause. When the precipitate was extracted with 80 per cent alcohol a portion of the lost potency was recovered. Heated at a pH of 4.0 there was no well marked precipitate; hence it was not filtered. The potency seemed even to be increased. Heated at pH of 6.2 and 7.2 precipitates formed, were filtered off, and extracted with alcohol, but no potency was recovered for the very obvious reason that none had been lost. The series proves plainly that heating to 80°C. for  $\frac{1}{2}$  hour does not destroy insulin and that this degree and duration of heat may be used for pasteurization when necessary.

Table II gives equally convincing evidence that heating to 75°C. in either 0.1 or 0.2 N HCl used as the extracting medium does no injury to the active substance and that heating to boiling in 0.2

N HCl may be borne as long as 20 minutes. The improved method of preparing insulin to be described in a later paper is based at the start upon these observations which are merely illustrative of many experiences in this laboratory.

TABLE II.  
*Effect on Potency of Heating in Process of Extraction.*

Preparation No.	Previous treatment	4 vols HCl	Heating		Rabbit test on first filtrate * Equivalent doses Drop in gm per 100 cc
			N	°C	
129 A	Ground 15 hrs.	0 2			0 035
129 B	" 15 "	0 2	75	Up to	0 074
129 C	" 15 "	0 2	75	½ hr.	0 070
129 D	" 15 "	0 2	75	1 hr.	0 030
134 A	" 15 "	0 1			0 033
134 B	" 15 "	0 1	75	Up to	0 040
134 C	" 15 "	0 1	75	½ hr.	0 050
134 D	" 15 "	0 1	75	1 hr	0 084
133 A	Not ground	0 2	75	Up to	0 076
133 B	" "	0 2	75	½ hr.	0 050
133 C	" "	0 2	75	1 hr.	0 076
133 D	" "	0 1	75	Up to	0 065
133 F	" "	0 1	75	1 hr.	0 056
135 A	" "	0 1	75	Up to	0 071
135 B	" "	0 1	75	½ hr	0 056
135 D	" "	0 2	75	Up to	0 074
135 E	" "	0 2	75	½ hr.	0 069
135 F	" "	0 2	75	1 hr	0 084
139 A	" "	0 2			0 051
139 B	" "	0 2	100	Up to	0 080
139 C	" "	0 2	100	10 min	0 053 Con-
139 D	" "	0 2	100	20 min	0 056 vulsions
139 E	" "	0 2	100	30 "	0 039

\* Neutralized to and tested at pH 4.1

3. *Dialysis*.—The insulin molecule, if it be a chemical entity, is evidently one of considerable size for it withstands dialysis in thin vegetable parchment for many hours. Table III gives ample support to this statement. Dialysis overnight has uniformly resulted in some loss of potency, but continued only from 4 to 15 hours it leaves the insulin behind and the shorter time suffices in most instances to remove diffusible salts.

TABLE III

*Effect of Dialysis upon the Potency of Extracts Given Subcutaneously to Rabbits and Diabetic Dogs.*

Before dialysis										After dialysis				
Date.	Animal	Weight	Extract No	Amount	Time inter- val	Blood sugar drop	Date	Animal	Weight	Extract No	Dialyzed	Amount	Time inter- val	Blood sugar drop.
1922		g		cc	hrs min		1922		g		hrs	cc	hrs min	
Oct. 6	Rabbit 2	2 3	68 (2)	10	2 30	0 035	Oct 26	Rabbit 2	2 3	68 (2)	15	10	2	0 031
83 Sept. 28	" 4	2 3	68 (2)	5	2 15	0 058	" 6	" 3	2 3	68 (2)	4	5	2	0 041
Oct. 4	" 7.	1 8	69 (2)	5	1 45	0 078	" 5	" 4	2 3	69 (2)	All night	12½	2 15	0 050
" 9	" 3.	2 3	69 (2)	10	3 30	0 024	" 4	" 2	2 3	69 (2)	4½	13	2 15	0 058
" 23	Dog 32.	8 5	77	25	4 15	0 158	" 24	Dog 32	8 5	77	Overnight	25	5 20	0 118
Nov. 7	" 36	10 3	84 (2)	10	4 5	0 104	Nov 8	" 36	10 3	84 (2)	4	10	4	0 153
" 9	" 36	10 3	85 (3)	10	4 25	0 173	" 10	" 36	10 3	85 (3)	4	4	6	0 126
														Convulsions. Blood at 0 021. Dog died.

4. *Adsorption*.—The following observations have been made upon the tendency of insulin to be withdrawn from solution by adhesion to solids in finely divided state. As already reported (9) we lost the potency from several preparations by attempting to clear them with Lloyd's reagent and with animal charcoal. In addition to these recorded experiences there was one other with Preparation 38 (2) of an entirely similar nature to that acquired with Dog 3 on July 18, 1922. After standing for some hours in contact with animal charcoal which had been previously treated with 95 per cent alcohol for the purpose of rendering it sterile, the filtrate, previously proved to be potent, produced no effect with Dog 19. In fact the blood sugar rose from 0.191 in 3 hours to 0.227. The extract at the time it was treated with charcoal, however, was in this case acid in reaction, somewhere between 0.2 and 0.25 N HCl, whereas in the previously reported case it was very faintly acid (pH of about 6.0).

This observation is in line with the contention of Mathews (17) that adsorption is really a kind of chemical reaction—in this instance between the acid insulin and the alkaline salts of the charcoal. This appears to be borne out by another experience that when the insulin extract is neutral it is not adsorbed by animal charcoal. Preparation 73 A was prepared on Oct. 4, 1922, from press-juice from 2 kilos of beef pancreas. 300 cc. of this juice were treated with 1,200 cc. of 95 per cent alcohol. After standing overnight it was filtered and concentrated in a vacuum still nearly to dryness. The residue, a yellowish fatty material, was rinsed from the flask with ether and chloroform and separated from the fat solvents by the addition of water. It was found to be neutral in reaction. An equal volume of 95 per cent alcohol was then added and the whole concentrated to 50 cc. A small portion of this was filtered off aseptically and used for test on a rabbit. The remainder was thoroughly mixed with animal charcoal and left filtering overnight. Tested on a rabbit next day the filtrate which was neutral in reaction showed more potency (evaporation) than before treatment with charcoal.

Preparation 125 was used also to demonstrate the ease with which insulin in aqueous solution may be removed from the solution by various adsorbents. The procedure and tests are indicated in Table IV.

Other tests have shown that only a small part of the insulin is removed from the charcoal or kaolin by extraction overnight with 80 per cent alcohol, whether applied neutral to litmus, acid to pH 4.1 with HCl, or distinctly alkaline with sodium hydroxide.

The difficulty of separating insulin from protein is due in no small degree to its adsorption by this class of substances. Many times in this laboratory it has been found that after precipitation in aqueous medium a quantity of insulin could be washed out of the precipitate by means of 70 to 80 per cent alcohol. Likewise it has been found (8) impossible except occasionally in percolates to extract from macerated pancreas as much insulin per unit mass of the tissue as can be washed out by perfusion. The chief cause of this difference seems to lie in the greater percentage of protein found in the extracts as compared with the perfusates. Percolates

TABLE IV.

*Adsorption Tests*

Lot 125, heated at 80°C for 1 hour, then concentrated in these fractions with alcohol.

Fraction	Concentrated	Test on rabbit, gm drop	Adsorbed at pH 4.1 on	Rabbit test	
				Filtrate	Hot water extract
A	1:16	-0.072	Lloyd's reagent	+0.013	+0.022
B	1:20	-0.047	Animal charcoal	+0.006	+0.013
C	1:20	-0.053	Kaolin	+0.009	+0.011

Subsequent treatment with hot water did not suffice to remove the insulin even when passed repeatedly through the adsorbent.

are intermediate in yield between perfusates and extracts as would be expected if the quantity of proteins present were the controlling factor. For in perfusion the cells are not broken at all or torn apart; in percolation of ground pancreas they are partly broken up; while in extraction, especially if stirring is employed, they are much more completely disintegrated and torn asunder, thus facilitating the solution of proteins.

*Chemical Properties.*

1. *Development of Potency.*—The hypoglycemic action of insulin varies with different conditions. Injected into a diabetic dog or normal rabbit in the form of the "first filtrate" (see page 323) it

has often given no reaction, or at most, a very feeble reaction either on the blood sugar or D:N ratio the 1st day, but the next day or

TABLE V.  
*Development of Potency in First Filtrate.*

Preparation No	Reaction		Condition Apr 21	Drop in blood sugar.	
	Mar 31	Apr 2		Mar 31 Dose = 10 gm	Apr. 2 pancreas
	N	N			
120(I)	0 0062	0 0116	Putrid odor	-0 038	Not given
120(II)	0 0101	0 0123	" "	-0 042	" "
120(III)	0 0141	0 0145	All right	-0 023	-0 060
120(III)	Neutralized to 0 0062 (Apr. 2).				-0 067
121(I)	This lot was neutralized to 0 0141 N and filtered. The filtrate was tested at once and at intervals Reaction did not change.				
	Date	Condition of filtrate	Test Dose = 10 gm pancreas		
	Apr. 3	Clear.	-0 016		
	" 4	"	-0 006		
	" 5	Turbid.	-0 064		
	" 7	"	-0 078 Convulsions.		
	" 12	Very turbid, precipitate in bottom of beaker, yeast odor, filtered, (yeast and bacteria present).	-0 080 "		
123	First filtrate, neutralized Apr 9 to 0 014 N and filtered The material was filtered overnight and tested daily.				
	Apr. 10	Clear.	-0 038 (2 hrs.)		
	" 11	"	-0 071 (3 " )		
	" 12	"	-0 075 (3 " )		
	" 13	Slight turbidity, fungus growth present	-0 057 (3 " )		
			-0 042 (3 " )		

the next the same solution without change of reaction may yield a satisfactory test. This apparent development of potency has been witnessed repeatedly and is illustrated in Table V. Unless

otherwise stated the rabbit test denotes the drop in blood sugar per 100 cc. of blood in 2 hours. These crude filtrates can be administered to animals by subcutaneous injection as previously reported (2) without any apparent toxic effect provided the acid employed in extraction is sufficiently neutralized. When brought to a pH of approximately 4.1 (titration to phenolphthalein of 0.014 normal) the filtrate after standing a few days (see Table V) becomes quite turbid and may show abundant growth of bacteria and yeasts without loss of potency to reduce the blood sugar. Even after the development of a strong odor of hydrogen sulfide the potency remains.

*2. Influence of Reaction on Potency.*—The exact reaction to which the neutralization is carried after extraction in 0.2 N HCl has much to do with the development of the hypoglycemic reaction in rabbits. Table VI contains several instances in which this effect of reaction is shown. Neutralizing to a pH of 5.0 to 5.7 (electrometric) or titration reaction to phenolphthalein of 0.005 to 0.009 N usually yields a satisfactory test at once. But if carried not quite so far (pH of 4.1 to 4.4, titration to 0.014 to 0.011 N)<sup>3</sup> the test is usually low, as noted above, the 1st day. This reaction, however, is favorable to rapid filtration and yields the clearest filtrate. Readjusted after filtration to pH 5.3 the test is much improved.

Concentration with alcohol as described on page 323 often develops the hypoglycemic reaction when it has been feeble or absent altogether in the first filtrate. Naturally the acidity increases in the process of distillation and it is probable that the stronger acid facilitates the liberation of insulin from the proteins. The tests, however, were made only after readjustment of the reaction to approximately pH 4.0, following dialysis for 4 hours. The dose given is equivalent in grams of pancreas represented, to the dose given as first filtrate, or to double this amount (Table VI). On the whole, first neutralization to pH 4.1 yields better results, notwithstanding the lower immediate test in the first filtrate, both because of more rapid filtration and because the acidity which develops in concentration liberates the active substance

<sup>3</sup> The difficulty of reaching an exact end-point in the presence of a considerable amount of protein will be appreciated. The titration reactions, therefore, are approximate only.

TABLE VI  
*Influence of Reaction (pH) on Potency Yield.*

Preparation No	Fraction No	Reaction		Filtration 6 hrs	Test of first filtrate, Dose = 10 gm pancreas	First concentration with 1 vol alcohol		Final concentration with 4 vols. alcohol	
		Titrated phenolphthalein	Electrometric pH			Ratio	Test, Dose = 20 gm pancreas.	Ratio	Test, Dose = 20 gm pancreas.
119	I	0 0036	6 63	cc 460 600 900	+0 001	1:12	+0 028	1:30	Lost.
	II	0 0062	5 32		-0 050	1 6 6	No blood.	1:30	-0 043
	III	0 0112	4 40		+0 001	1.9 2	-0 040	1:30	-0 037
120	I	0 0043	6 17	320 (1 hr )	-0 038	1:8	No blood	1:40	-0 056
	II	0 0087	5 04		-0 042	1:6 8	-0 055	1:40	-0 052
	III	0 0119	4 29		-0 023	1:6 8	Not tested.	1:40	-0 016
121	III readjusted	0 006	5 32	Rapid. Good Better Best	-0 057				-0 025
	I	0 014	4 11		-0 016	1 5	-0 071		= 10 gm.
	II	0 0054	6 08		+0 019	1.5 1	Not tested	1 20	+0 003
122	III	0 009	5 50(?)		-0 040	1:7 4	" "	1:20	-0 016
	IV	0 0137	4 21		±0 0		" "		-0 014
	IV readjusted	0 006	5 32		-0 017		10 gm.		-0 021
122	I	0 0065	5 70		-0 061	1:7 1	Not tested	1:20	Not tested.
					-0 028				= 20 gm. -0.036
	II	0 009	5 02		-0 021	1:8	-0 012	1:20	= 10 gm. -0 004 ±0 0
122	III	0 014	4 18		-0 037		-0 031	1:20	-0 049
					+0 006	1:8	Not tested	1:20	-0 024
					-0 039				-0 043 -0 021



better. This reaction also is well outside the isoelectric point (see page 324).

3. *Stability*.—Insulin seems to be a fairly stable compound. In its purest form we have yet to note any deterioration as judged by its hypoglycemic reaction in normal rabbits. Purified of proteins as far as this can be done by means of alcohol (see method on page 323) it has been kept for as long as 4 months. At the end of this time the reaction was 0.03 N HCl. The reaction at the start was not recorded, but there is no reason to believe it had

TABLE VII  
*Influence of Reaction on Stability.*

Preparation	No 98 (2)	Concentration 1 40	Three $\frac{1}{2}$ oz bottles
Fraction	(1)	(2)	(3)
Original acidity	0 1 N HCl	0 08 N HCl	0 06 N HCl
Acidity after 11 days	0 097	0 087	0 063
Rabbit tests	-0 019 gm	-0 063 (Con- vulsions )	-0 051 (Con- vulsions )
	-0 040 "	-0 048 (No con- vulsions)	-0 043
Preparation	No 99 (2)	Concentration 1 40	
Fraction	(1)	(2)	(3)
Reaction pH	5 7	6 7	7 7
Rabbit tests after 1 wk . . .	-0 094	-0 030	-0 041

changed materially, as it was preserved with 0.1 per cent tricresol. In this form insulin does not appear to keep quite so well at a reaction of 0.1 N HCl as at a reaction of 0.06 N (Table VII). The lowest acidity at which it has been observed to keep well is pH 5.7. At or above the neutral point there was distinct deterioration.

4. *The Chemical Nature of Insulin*.—It must have occurred to many readers of the recent literature on insulin that it presents some physiological features in common with Hopkins' (18) "glutathione"—that it might be a sort of activated glutathione concentrated by some selective action of the islet cells. The method of extraction which had yielded the best results in our hands (19) proved quite unconsciously to us, to be similar in a general way to that employed by Hopkins in extracting his dipeptide from

animal tissues. The solubility of insulin, as obtained by acid extraction, in water and its nearly total insolubility in organic reagents like absolute alcohol and ether, and its greater stability in acid media than in alkaline are additional points of similarity. Crude aqueous solutions of insulin, like our "first filtrate," very readily undergo spoilage with liberation of abundance of hydrogen sulfide. Very naturally, therefore, we have repeatedly tried to demonstrate the presence of sulfur in the sulfydryl form by the nitroprusside reaction. The purest product obtained as judged by the biological tests and by freedom from proteins has steadily refused to give this reaction even after exposure to nascent hydrogen or to the reducing action of  $\text{SO}_2$ .

Heated with lead acetate and  $\text{NaOH}$  there is a slight browning of the fluid which perhaps indicates the presence of a trace of sulfur as an impurity. Certainly there is not a sufficient amount of sulfur in this pure product to account for the very large evolution of hydrogen sulfide from crude products. It must have its source in the proteins. If sulfur is present in the oxidized form of a double sulfide the compound must be exceedingly stable.

Insulin, therefore, is not a concentrated form of glutathione. Since the latter is present in all tissues examined by Hopkins and certainly is present in the fresh pancreas itself if a vivid reaction to nitroprusside may be taken as proof, it would scarcely be necessary in a compound whose chief function appears to be to prepare glucose for combustion. The observations of Macleod (20) and of Cori, Pucher, and Cori (21) on the influence of insulin on glycogen formation indicate that it is essential here. The latter observers found that insulin would inaugurate the formation of glycogen in the rabbit's liver when the free sugar of the liver is below its normal threshold. The observation of Winter and Smith (22) that insulin acting with some hormone in the liver can transform glucose of the ordinary variety into glucose which is, according to them, the form in which glucose probably occurs in the blood, gives strength to this conception. The mechanism of oxidation is already present in the tissues (glutathione); it requires a special product (insulin) with immediate access to the portal system to catch glucose as it enters the circulation and in cooperation with the liver to transform it into an oxidizable form.

In a subsequent paper we shall report the results of analyses of the purest product which we have been able to obtain.

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## AQUEOUS EXTRACTS OF PANCREAS.

### III. SOME PRECIPITATION REACTIONS OF INSULIN.

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Rapid preparation of insulin for medicinal purposes requires that a means be found of removing it from solution by some method more expeditious than that of drying. The previous study was convincing regarding the necessary conditions for rapid extraction. Avoidance of expense obviously favors an aqueous method, but the task of effecting the necessary concentration for use in human cases even by vacuum distillation is time-consuming. The present study was undertaken with a double purpose: (1) to find an inexpensive method of concentration and (2) to take the preliminary steps toward isolation of the pure substance. It was begun in October, 1922, and has been pursued as opportunity afforded the use of potent preparations not required for more urgent purposes.

The results here presented should be regarded as preliminary, but will be found, we think, both reliable and consistent. They have led to the development of the improved method of preparation to be described in a subsequent paper.

The preparations used were the purest available by the methods in use up to April of this year. For the most part these methods varied little from that described in the previous paper (Paper II of this series). All these preparations gave the biuret reaction varying from feeble to vivid, and it is quite possible that the precipitation recorded in many instances is in part a reaction with protein as well as with the insulin itself. Nevertheless, the protein was the least in amount we were able to attain and can scarcely have been the only source of the precipitate, for many of the reagents which failed are well known protein

precipitants. Even if it were the sole cause, the reactions recorded should be of value in the evolution of other methods.

The list of reagents which were successful fall into two general classes: organic and inorganic; but in presenting the results we shall subdivide the former into two divisions, the alcohols and other organics. The following were found to be wholly ineffective as precipitants or else proved to be impracticable as aids to a rapid method because of the difficulty of removing the excess: ethyl acetate, pyrogallol, tannic acid, benzene, formaldehyde, and carbon tetrachloride among the organic reagents available; and phosphotungstic acid, phosphomolybdic acid, uranium acetate, zinc sulfate, copper sulfate, ferric chloride, lead acetate, mercuric chloride, and potassium mercuric iodide among the inorganic reagents.

#### *Method of Procedure.*

In order to make the reactions more or less comparative a procedure as nearly uniform as possible was adopted. This consisted in adding a specific amount of the reagent, throwing down the precipitate by centrifuge, taking up in distilled water, and testing as soon as possible on rabbits. The supernatant liquid was dried with or without dialysis, depending upon the nature of the additive, and similarly dissolved and tested (Filtrate in tables). The amount of material employed for test was equivalent, in grams of pancreas represented, to the amount of the original extract tested.

#### *Results.*

The results expressed by the tables do not include many little points of behavior which observed and noted at the time often are of more significance than the actual figures obtained. To record all these details at this time would be merely confusing. Only such results are given as there is good reason to believe would be of value in following up the use of the precipitant for purposes of isolation. Only preparations known to be potent are incorporated in the table. Many data had to be discarded because subsequent test proved the extract impotent or very weak.

Mechanical difficulty has ruled out a number of substances not mentioned in the table. This difficulty is of two kinds: immiscibility of the extract and reagent and unusual difficulty in

TABLE I.  
*Precipitation Reactions with Alcohols.*

Reagent	Vols	Preparation No	Blood sugar reaction		
			Original	Precipitate	Filtrate
Absolute alcohol	5	84(2)	-0 045	+0 017	-0 085
“ “	5	75(2)	-0 061	-0 010	-0 070
Methyl “	5	120(I)	-0 056	-0 033	+0 003
Absolute ethyl alcohol	5}	120(I)	-0 056	-0 057	
Methyl alcohol	4}				
<i>n</i> -Butyl “	5	101AB	-0 076	-0 075	?
“ “	5	105	-0 087	-0 064	+0 013
Amyl “	5	101AB	-0 076	-0 082	+0 003
Absolute “	5}	106B	-0 028	-0 039	-0 003
<i>n</i> -Butyl “	4}				
Absolute “	4}	106	-0 069	-0 053	+0 014
<i>n</i> -Butyl “	5}				
Absolute “	4}	120(I)	-0 056	-0 056	
<i>n</i> -Butyl “	5}				
Absolute “	5}	125	-0 038	-0 003	
<i>n</i> -Butyl “	5}				
Absolute “	5}	106B	-0 028	-0 052	
Amyl “	4}				
Absolute “	5}	108	-0 069	-0 039	
Amyl “	4}				
Absolute “	5}	125	-0 038	-0 080	
Amyl “	5}				
Capryl “	5	101AB	-0 076	-0 005	
Absolute “	5}	106B	-0 028	-0 050	
Capryl “	4}				
Absolute “	5}	120(I)	-0 056	-0 041	
Isopropyl “	4}				

TABLE I—*Concluded.*

Reagent		Vols	Preparation No	Blood sugar reaction.		
				Original.	Precipitate	Filtrate
Absolute alcohol	.	5}	120(I)	-0 056	-0 061	
<i>n</i> -Propyl	"	4}				
Absolute	"	5}	119(III)	-0 037	-0 068	
<i>n</i> -Propyl	"	4}				
Absolute	"	5}	125	-0 038	-0 001	+0 002
<i>n</i> -Propyl	"	5}				

removing the excess of reagent. The first of these has latterly proved far from a hindrance and indeed, a decided aid, for using materials on a larger scale, as in the precipitation with amyl alcohol, it is found that the precipitate will form an agglutinative layer between the aqueous and alcoholic layers, so coherent that both the liquids may be withdrawn without disturbing it. The difficulty of removing reagents on the other hand is more acute. While it would be going too far to say that many of the reagents, especially salts of the heavy metals could not be removed without destruction of the active principle, the present purpose of finding an easily removable reagent does warrant the classification of such as will not submit to dialysis or to evaporation under the heading of mechanical interference. Attempts to use salts of heavy metals such as lead and iron as precipitants of proteins instead of alcohol led to many discouragements at an earlier stage of our studies, mainly, it is supposed, because of the adsorbent properties of insulin (see previous paper).

1. *Alcohols.*—All the alcohols used give precipitation reactions with the insulin extracts. When the alcohol was added in the proportion of 5 volumes to 1 of the aqueous extract, making 83 per cent by volume of the alcohol, the precipitation was rarely complete. Normal butyl and amyl alcohols are exceptions, both yielding in many trials, in addition to those shown in the table, all the potency in the precipitate. Adding first to the aqueous extract 4 or 5 volumes of absolute alcohol (making 80 to 83 per cent) which in this proportion throws down but little of the potency, the insulin was almost invariably recovered in

the precipitate whatever the alcohol subsequently added. The two alcohols together made up approximately 90 per cent by volume. There seems to be something specific about the reaction of insulin with the alcoholic hydroxyl. In fact, the potency of the precipitate so obtained seems often to be distinctly augmented. The precipitate is invariably soluble in water with great ease.

Because of its peculiar property to throw out insulin as an insoluble layer between the aqueous and alcoholic layers even when added in relatively small amount, amyl alcohol has become

TABLE II.  
*Precipitation Reactions with Other Organic Reagents.*

Reagent	Vols	Preparation No.	Blood sugar reaction.		
			Original	Precipitate	Filtrate
Acetone	5	84(2)	-0 045	-0 034	0 00
	5	75(3)	-0 061	+0 038	-0 009
	5	87(3)	-0 047	-0 038	-0 009
	5	Same reprecipitated		-0 049	
	5	93(2)	-0 067	-0 120	+0 004
Ether	5	84(2)	-0 045	-0 031	-0 053
	5	75(3)	-0 061	+0 011	-0 018
Chloroform.	5	84(2)	-0 045	-0 013	-0 004
	5	75(3)	-0 061	-0 015	-0 001
Toluene.	5	84(2)	-0 045	-0 003	-0 029
Xylene	5	84(2)	-0 045	?	-0 067
Trichloroacetic acid.	5-3 per cent	105	-0 087	-0 069	+0 003
	5-3 " "	108	-0 069	+0 012	-0 026
	5-3 " "	108 v	-0 069	-0 020	-0 018
	5-2 5 " "	120(III)	-0 061	-0 002	
	1-20 " "	125	-0 038	-0 039	

a favorite in this laboratory and it may be recommended on the following grounds: (1) its cost is approximately the same as other higher alcohols; (2) due to its high boiling point it is more effectively recovered by fractional distillation; (3) its precipitates are as potent as those of other alcohols; and (4) because of its slight solubility in water its addition to a 70 or 80 per cent ethyl alcohol



solution separates sufficient water to dissolve some salt and leave the intervening layer free from this impurity. Normal butyl alcohol which has some properties in common with amyl as regards its reaction with insulin has not proved quite so practicable in manipulation.

2. *Other Organic Reagents.*—Acetone acts in many respects similar to the higher alcohols, but is not so dependable. Moreover, it throws down a substance, soluble in 95 per cent alcohol which has a distinctly hyperglycemic effect. Our first observation of this fact was made early in December, 1922, when an extract of beef pancreas in 0.2 N HCl, which had been purified of proteins partly by neutralization of the acid (metaproteins) and partly by precipitation with 5 volumes of 95 per cent alcohol, concentrated to one-tenth volume, defatted with chloroform, concentrated again with an equal volume of alcohol, dialyzed for 18 hours, was precipitated with 5 volumes of c.p. acetone. After standing 4 hours this precipitate was caught on paper and washed thoroughly with 95 per cent alcohol. The alcohol was removed by vacuum distillation and the residual extract taken up in sterile water. Given by subcutaneous injection to two depancreatized dogs it raised the blood sugar in 3 hours as follows:

Dog 1	0 380 to 0.590.
Dog 2	0 140 " 0 390.

4 days later the material was tested on normal rabbits with the same, though smaller effects. That portion of the acetone precipitate which was insoluble in 95 per cent alcohol contained an abundance of insulin. This observation has been repeated several times in all essential details. This hyperglycemic substance has been given the name *glucagon* and its properties will be described more fully in a later paper. It is mentioned here because by the method described above it has been separated also from cat's muscle and from yeast.

A series of experiments was conducted with acetone as a precipitant with three equivalent portions, one made slightly alkaline, one neutral, and the third slightly acid. In those products which gave positive tests there was a very marked gradation first in the potency and second in the nitrogen content. The acidified portion yielded the most potent precipitate and had the lowest nitrogen content.

Precipitation tests with ether, chloroform, toluene, and xylene were made with the object of determining whether these agents may not at times carry down a considerable amount of the insulin when employed for removal of fats. The tests here recorded confirm other observations made in this laboratory and emphasize

TABLE III  
*Effect of Reaction on Precipitation by Acetone*

Extract No	Precipitant	Reaction or pH	Blood sugar reaction		Nitrogen (in precipitate)
			Original potency	Potency of precipitate	
98(2)	Acetone	Slightly acid	-0 090	-0 039	6 5
98(2)	"	Neutral.	-0 090	-0 039	
98(2)	"	Slightly alkaline.	-0 090	-0 023	
99(2)A	"	5 8	-0 063	-0 094 -0 071	4 26
99(2)A	"	6 7	-0 063	-0 030 -0 023	4 92
99(2)A	"	8 8	-0 063	-0 041 -0 053	6 08
103(1)	"	5.7	-0 046	+0 007 -0 008	10 3
103(1)	"	6 7	-0 046	+0 004 -0 003	10 2
103(1)	"	7 8	-0 046	+0 015 -0 028	10 0
105	"	5 7	-0 087	-0 017	
105	"	6 7	-0 087	-0 011	
105	"	8 7	-0 087	-0 013	

the advantage of aqueous methods of extraction as against alcoholic. Xylene seems to be the least dangerous from this point of view, but it is not a particularly efficient fat solvent.

The status of trichloroacetic acid has been and still is, difficult to formulate. Its use in small concentration has seemed most hopeful, as the precipitate formed is very small in amount.

Unfortunately, however, this has not always contained potency. Accordingly, while its use is to be recommended the conditions of its application are far from determined.

3. *Salts*.—Of the salts which are commonly used in salting out proteins, only ammonium sulfate and sodium chloride have accomplished the desired results. The former has not in our hands given as encouraging results as Shaffer and his colleagues would lead one to expect. Sodium chloride has given excellent results. The first successful use of this reagent was by the addition of 9 volumes of a saturated solution to 1 of the purified extract, but in order to avoid such large volumes of liquid, solid salt was

TABLE IV  
*Precipitation with Salts*

Reagent	Amount used	Preparation No	Blood sugar reaction	
			Original	Precipitate
Ammonium sulfate	$\frac{1}{3}$ saturation	119(III)	-0 037	-0 024
	$\frac{4}{5}$ "	108 x	-0 068	+0 079
	$\frac{9}{10}$ "	119(III)	-0 037	-0 014
Magnesium sulfate	$\frac{1}{2}$ "	119(III)	-0 037	-0 005
	$\frac{1^9}{20}$ "	119(III)	-0 037	No precipitate.
Sodium sulfate	$\frac{1}{2}$ "	119(III)	-0 037	$\pm 0$ 00
	$\frac{1^9}{20}$ "	119(III)	-0 037	+0 010
Sodium chloride	$\frac{1}{2}$ "	119(III)	-0 037	+0 012
	$\frac{9}{10}$ "	119(III)	-0 037	-0 051
	Saturation	125	-0 076	-0 084
	"	125	-0 076	-0 076

added in quantity sufficient to saturate; *i.e.*, 35 gm. per 100 cc. The precipitation of insulin is complete. Filtrates have been repeatedly tested with negative results. As will be more fully discussed in another paper this reagent more nearly satisfies the object of this study than any other. It is inexpensive, is readily removed, but if traces are left it does no harm to the organism. When ammonium sulfate is not completely removed its injection may cause a hyperglycemic reaction. Sodium chloride up to 3 per cent has not caused such a reaction.

A number of other instances of hyperglycemic reaction far beyond any experimental error have been encountered in the course of this work and especially in connection with the use of salts. One in particular is worthy of mention because we have not encountered its record in the literature of blood sugar studies. Using cadmium chloride in an attempt to remove lecithin from alcoholic extracts a certain filtrate containing the salt gave a rise of 339 mg. against an original drop of 96 mg.

Inasmuch as the antidiabetic substance was not thrown out of solution by ethyl acetate, it was thought there might be a possibility of the latter having a solvent action. It was also possible that even if the insulin were not dissolved out, some impurity might be, and as the object of isolation is to remove impurities either in groups or piecemeal, the use of any solvent is warrantable. The material extracted with ethyl acetate has given very conflicting results, the change in blood sugar ranging from  $-0.061$  to  $+0.024$ . Hence, conclusions are not yet to be drawn. The amount extracted is small, but we have had the satisfaction of noting the first distinct crystalline formation in connection with this work. Not only this, but there were three distinct types present. The first two of these possessed a yellowish color, probably due to an impurity in the ethyl acetate, for on washing with a small amount of the latter or with water, the yellow could be more or less removed. The ethyl acetate itself on evaporation left a yellow oily residue but no crystals. After several weeks the crystalline structure disappeared and while it is not felt that there is yet evidence that any of these are pure insulin, it is nevertheless advisable to mention the fact of their presence.

#### SUMMARY.

By way of summary, it is perhaps best to draw up a list of the reagents used as precipitants and group them under these heads—no precipitate, interfering circumstances, negative (this term implies less finality than “destructive”), and positive, thus:

*No precipitate.*  
Formaldehyde.  
Ethyl acetate  
Benzene.

*Interfering circumstance*  
Pyrogallol.  
Picric acid  
Phenylhydrazine

Toluene.  
Carbon tetrachloride.  
Ferric chloride.  
Copper sulfate.  
Magnesium sulfate.  
Chloroform ?

*Positive.*

Ammonium sulfate.  
Sodium chloride.  
Trichloroacetic acid  
Acetone.  
Alcohol.  
Methyl.  
Ethyl.  
Isopropyl.  
*n*-Butyl  
Isoamyl.  
Caprylic

Copper acetate.  
Basic lead acetate.  
Petroleum ether ?

*Negative.*

Cadmium chloride.  
Phosphotungstic acid.  
Sodium sulfate.  
Zinc sulfate.  
Phosphomolybdic acid.  
Uranium acetate.  
Sodium acetate  
Mercuric chloride.  
Potassium mercuric iodide  
Bromine.  
Ether ?

With regard to the properties of insulin as it has been observed in this laboratory, not much can be said. It is a white, amorphous powder probably insoluble in neutral water when pure. It gives no protein reactions of any kind, and the most potent that have been analyzed have had a low nitrogen content, 4 to 6 per cent dry weight.

## THE QUALITATIVE TESTS FOR ACETONE BODIES; THEIR SIGNIFICANCE AND VALUE.

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The qualitative tests most commonly used for acetone bodies in the urine are the sodium nitroprusside test and the ferric chloride test. These are usually regarded as selective tests for acetone and diacetic acids, respectively. In fact, few text-books on methods of analysis mention the sodium nitroprusside test as a test for diacetic acid, while some workers—Harding and Ruttan (1) and Hunter (2)—regard it as probably selective for diacetic acid alone. Occasionally, in case reports these qualitative tests have been allotted an unwarranted quantitative significance. Both tests are known to be interfered with or rendered positive by other substances.

For these reasons and because there is contradictory evidence in the literature on the subject, we took the opportunity afforded in a metabolic study of a series of cases of diabetes to make both qualitative and quantitative analyses on the same samples of many specimens of urine. In addition we have carried out certain experiments devised to throw light upon the selectivity, sensitivity, and quantitative significance of these tests, in some instances repeating the work of others.

In 1882, Legal found that sodium nitroprusside in the presence of sodium or potassium hydroxide produces a ruby-red color in a solution containing acetone or creatinine. Coincidentally, le Nobel (3) observed that if acetic acid is added to the solution before the base, the color changes to purple (sometimes spoken of as violet or a permanganate tint), and that this occurs only when the solution contains acetone; it is not produced by creatinine.

## 348 Qualitative Tests for Acetone Bodies

In 1908, Rothera (4) pointed out that ammonium hydroxide is more satisfactory than sodium or potassium hydroxide, and that the test is more sensitive in the presence of ammonium sulfate.

Harding and Ruttan (1) investigated the sodium nitroprusside reaction and conclude that it detects diacetic acid rather than acetone; that since an aqueous solution of free acetone shows a less marked Legal reaction than does diabetic urine containing the same amount of acetone, there must be some other substance in diabetic urine which favors the appearance of the purple ring; that diabetic urine saturated with NaCl, and freed from acetone by aeration gives a positive sodium nitroprusside test to dilutions of 1 to 30,000 and a positive ferric chloride test to dilutions of 1 to 7,000. Hydrolyzed diacetic ester gives a positive nitroprusside test to a dilution of 1 to 80,000.

### EXPERIMENTAL.

The following is the technique used.

*Sodium Nitroprusside Test.*—Transfer about 10 cc. of urine to a test-tube and add 20 drops of the following solution. Glacial acetic acid, 10 cc., + 10 cc. of a 10 per cent solution of Na nitroprusside. (This solution will keep well for at least 2 weeks in a brown glass bottle.) Shake the test-tube until the specimen is well mixed, and then layer on this 1 to 2 cc. of concentrated  $\text{NH}_4\text{OH}$  solution. At the contact of the fluids, a purple ring appears. After standing 2 minutes, the reading is made and recorded. In order to standardize these readings roughly, we record them as follows:

Trace	The ring is very faint.
+	Small, but definite ring
++	Large ring.
+++	Very large ring
++++	Heavy purple mass

*Ferric Chloride Test.*—10 cc. of a 10 per cent solution of ferric chloride ( $\text{FeCl}_3$ ) are added to 10 cc. of urine, more is added, if necessary, to clear the precipitate. The color appears instantaneously and the reading can be immediately made. For approximate comparison, the readings are recorded as follows:

Trace	Light brown.
+	Dark brown.
++	Light burgundy.
+++	Dark burgundy.
++++	Black.

*Data Concerning the Selectivity of the Qualitative Tests.*

1. *Acetoacetic ethyl ester* gives a positive ferric chloride test and a negative sodium nitroprusside test.

2. These two tests could not be made on *butyric ethyl ester* because it does not mix with water. Attempts to test the substance when water was added and the mixture shaken gave negative results with each test.

3. When applied to *free acetone solutions*, the ferric chloride test is negative, even with chemically pure acetone (not acid to litmus). The nitroprusside test is positive.

In order to be certain that this acetone solution contained no other acetone bodies, *i.e.* diacetic acid, we determined quantitatively the content of acetone by Folin's method for free acetone alone, and by Van Slyke's method for total acetone bodies, expressed in grams of acetone. These determinations were made on a 1 per cent by volume solution. The results follow.

	gm
By Van Slyke's method	0 674
	0 716
Average . . . . .	0 695
By Folin's method . . . . .	0 698
	0 700
Average.....	0 699

A 0.1 per cent solution by volume represents 1 cc. of acetone per liter. As 0.79 is the specific gravity of acetone, and the experiment was made at 25°C., 0.7 gm. of acetone is within the limits of error. Since the determinations of acetone by the two methods agree, it is obvious that no other acetone bodies, such as diacetic acid, were present in the solution.

A 10 per cent solution by volume of acetone was kept for 2 weeks in the ice box in a glass stoppered flask. The above qualitative tests were then repeated with the same results as before.

4. *Diacetic acid* was prepared by hydrolyzing diacetic ethyl ester according to the method of Harding and Ruttan (1). A little HCl was then added to the solution, to bring its pH approximately to that of the average reaction of urine. This solution



was kept at room temperature in a closed flask and analyzed at intervals of a few days.

The results of the analyses were as follows:

1st day.

Ferric chloride test	++++
Nitroprusside test	+++
Diacetic acid + acetone expressed as grams of acetone.	5.56
Free acetone	0.29

Diacetic acid (by difference of the two)	5.27
--	------

All determinations were made in duplicate and checked within the limits of error of the methods. As 64 is the molecular weight of acetone, and 142, that of the ester, 13 gm. of ester expressed in acetone will be equal to  $13 \times \frac{64}{142}$  or 5.85 gm. Since the diacetic ester was roughly weighed, 5.56 checks well with the expected 5.85.

Hydrolysis of ester was probably completed by the procedure of the Van Slyke method.

On the 2nd, 3rd, 4th, and 5th days, the qualitative tests remained the same. On the 5th day, a sample was withdrawn and treated by Folin's method for free acetone determinations; that is, 10 drops of a 1 per cent solution of  $\text{H}_3\text{PO}_4$ , excess of  $\text{NaCl}$ , and a little kerosene were added, and the mixture was aerated for 40 minutes. The qualitative tests on this solution were as follows:

Ferric chloride test	+	(or considerably decreased).
Nitroprusside test	++++	(or increased).

The solution was again aerated, but this time through a known amount of 0.1 N iodine solution, in order to test whether the first aeration period had been continued long enough to carry off all the free acetone. Some of the iodine was converted into iodoform, showing that some acetone still remained. The period of aeration was therefore repeated and iodine titration proved the absence of all free acetone. The qualitative tests were applied and found to be + for ferric chloride and +++++ for Na nitroprusside. The diacetic acid by quantitative analysis proved to be 2.75 gm. per liter, the original concentration having been 5.56 gm. per liter.

Thus we have a solution containing less than half the original amount of acetoacetic acid and no free acetone, and yet the qualitative test with sodium nitroprusside is stronger than in the original solution. *This suggests that the nitroprusside test is positive when diacetic acid alone is present.* But why there should be a more intense reaction with half as much diacetic acid present, was difficult to say. (See discussion under sensitivity of the tests.)

In order to test further the selectivity of sodium nitroprusside for diacetic acid, we carried out the following experiments.

*Experiment 1.*—A 100 cc. specimen of solution of hydrolyzed ester, withdrawn from the stock solution as it reached the 5th day of hydrolysis (see Table III), was boiled again under reflux condenser. The flask was not removed from the reflux condenser until it had cooled. The volume of the fluid remained the same. Both qualitative tests were again negative. The solution contained acetone only, because Van Slyke's method for diacetic acid + acetone showed 0.280 gm. per liter, and Folin's method for free acetone showed 0.293 gm. per liter (that is, the same result in both determinations); hence all the diacetic acid had disappeared. If it was converted into acetone, most of this had disappeared too, because the concentration of free acetone remained unchanged, in spite of which fact the sodium nitroprusside test was strongly positive (+++) before boiling, and negative after boiling. *This can only be explained by the disappearance of the diacetic acid*, since the amount of free acetone left in solution is too dilute to be capable of producing a positive nitroprusside test. (See Table II.)

*Experiment 2.*—A 50 cc. sample (accurately withdrawn with a pipette) of the specimen corresponding to the 5th day of hydrolysis was aerated for 40 minutes after adding 10 drops of a 10 per cent solution of  $\text{H}_3\text{PO}_4$ , excess of  $\text{NaCl}$ , and some kerosene in order to free the specimen from acetone (Folin's procedure). 10 cc. were then pipetted for qualitative tests.

Ferric chloride . . . 0 (it was positive before aeration).

Nitroprusside. . . ++++ (it was only +++ before aeration).

The 40 cc. left were aerated again for 20 minutes, through 10 cc. of 0.1 N iodine solution. The excess of iodine was titrated with

0.1 N Na thiosulfate in the presence of starch, and showed no free acetone in the solution after the first aeration period since exactly 10 cc. of iodine were recovered. The total acetone bodies in the remaining solution were determined with Van Slyke's method (5). The result was a concentration of 0.138 gm. per liter, which evidently represented nothing but diacetic acid (initial concentration of diacetic acid before aeration being 0.530 gm. per liter). Aeration has then removed much of the diacetic acid in addition to the acetone. What happens to the former, we do not know. However, the nitroprusside test is increased, at least not decreased, even when diacetic acid is decreased.

That sodium nitroprusside detects diacetic acid is obvious from the fact that it remains positive, although quantitatively there is no free acetone left in solution.

The purple ring in this solution, saturated with NaCl, instead of fading on long standing, as is usual, changes readily and steadily into a heavy, nearly black mass, as if the substance responsible for the positive test were gradually accumulating around the level of the ring in the test-tube. This, however, is not the case, because after standing 1 hour, the clear, colorless fluid at the bottom of the test-tube, beneath the level of the purple ring, on being pipetted out, poured into a fresh tube, and tested again with nitroprusside, showed a new ring, just as increasingly heavy as the first one.

We were not satisfied that these experiments showed that nitroprusside in contact with diacetic acid formed a purple ring in the absence of any trace of acetone; since we had added other substances to the solution, any one of several of which might clearly influence the test. In order to prove our assumption it was necessary, therefore, to repeat our experiment of this removal of all free acetone without the presence of any additional substances.

The solution used was that of the 8th day of hydrolysis (see Table III). 25 cc. of this solution plus 25 cc. of  $H_2O$  (both accurately pipetted) were aerated for 4 hours. The total volume remained 50 cc. at the end of the period of aeration. The ferric chloride test on this mixture gave a negative result (there was a trace before aerating) while the nitroprusside test gave a + reading (it was +++ before aerating).

	<i>gm. per liter</i>
Diacetic acid + acetone. . . . .	0 056
Free acetone (Folin's method) . . . . .	0
Diacetic acid . . . . .	0 056

Hence, a 4 hour period of aeration is sufficient to remove all free acetone from a dilute solution in the absence of NaCl (less than 4 hours did not remove all free acetone). However, the nitroprusside test remains positive, and must be due to the small amount of diacetic acid present in the mixture (0.056 gm. per liter).

In order to rule out the possibility that the procedure of the sodium nitroprusside test might decompose some diacetic acid into acetone sufficiently to render the latter responsible for the appearance of the purple ring, the following experiment was performed.

A solution containing diacetic acid and acetone was saturated with NaCl and aerated through a known amount of iodine. The concentration of free acetone proved to be *0.402 gm. per liter*. The same titration was repeated on a second sample of the solution, but Na nitroprusside, acetic acid, and  $\text{NH}_3$  were added in the same proportion as those for Legal's test. The color of the mixture became dark purple. It was left standing for  $\frac{1}{2}$  hour under kerosene, and then aerated through iodine. The titration showed a concentration of *0.395 gm. per liter*. Hence, we conclude that no free acetone is liberated from a diacetic acid solution, when tested for ketone bodies with Na nitroprusside, acetic acid, and ammonia. *Therefore, there is no doubt that diacetic acid alone may give a positive nitroprusside test.*

#### *Data Regarding the Sensitivity of the Qualitative Tests.*

The intensity of the reaction of the ferric chloride test to different dilutions of the *diacetic ethyl ester*, is given in Table I.

The intensity of the sodium nitroprusside tests, obtained with various concentrations of pure acetone solutions, is presented in Table II.

We conclude from this that a pure aqueous solution of acetone, free from diacetic acid, gives a nitroprusside test to dilutions of 1:1,000.

With diacetic acid solution, the sodium nitroprusside test showed a positive reaction to a dilution of 1:20,000. At this grade of dilution, the ferric chloride test is not positive.

It is interesting, moreover, to compare this with the findings on acetone solutions of Table II, which show that at the corresponding grade of dilution of acetone, the test is not positive. It is also serviceable to compare these figures with those found in a fresh specimen of urine, which contained no acetone, and some

TABLE I

Concentration of acetoacetic ethyl ester	Color reaction with FeCl <sub>3</sub> .	Symbol
<i>per cent</i>		
0.1	Yellow.	0
0.2	Light brown.	Trace.
0.25	Dark	+
0.3	" "	+
0.5	Light burgundy	++
1.0	Dark	+++
More than 1.0 per cent by volume.	Black	++++

TABLE II

Dilution by volume	Solvent		
	H <sub>2</sub> O	Normal urine	Diabetic urine sugar-free and both acetone bodies tested negative.
<i>per cent</i>			
0.0001	0	0	0
0.001	0	Very faint trace	Very faint trace ?
0.01	0	Trace	Trace.
0.1	Trace.	+	+
1	+	++	++
10	++	+++	+++

diacetic acid (0.076 gm. per liter). Both qualitative tests were slightly positive. This would suggest that the limit of dilution for which a nitroprusside test is still positive would be about 1 to 15,000, whereas, in our solution of pure diacetic acid, acetone-free, the dilution of which was greater (1 to 20,000), the nitroprusside test was still definitely positive. Apparently, then, the Na nitroprusside test is less sensitive for acetone than for diacetic acid.

It was noted on page 351 that we had a solution "containing less than half the original amount of acetoacetic acid and no free acetone, and yet the qualitative test with sodium nitroprusside is stronger than in the original solution." In the hope of learning the reason for this discrepancy, we repeated the experiment, using less diacetic ethyl ester, in order to obtain proportions of ketone bodies which correspond to the usual amounts found in

TABLE III.

*Repeated Analysis, at 2 or 3 Day Intervals, of a Mixture of Diacetic Acid and Acetone Obtained by Hydrolysis of Ethyl Diacetate.*

Days of hydrolysis.	Ferric chloride test.	Nitroprusside test.	Total acetone bodies Acetone + diacetic acid expressed as acetone (Van Slyke method).	Duplicates of weights of precipitates for 2.5 cc. of solution.	Free acetone (Folin method).	Duplicates of free acetone determinations	Diacetic acid expressed in acetone Difference of total acetone bodies and free acetone.
			gm. per liter	gm.	gm. per liter		gm.
1	+++	++	0 720	0.0365	0 105	0 10	0 615
				0 0352		0 11	
3	+	+++	0 750	0 0380	0 175	0 187	0.575
	Dark brown.			0 0372		0 163	
5	Trace. Light brown.	+++	0 80	0 0398	0 270	Duplicated check within 1 drop of 0.1N thio-sulfate.	0 53
				0 0406			
8	Trace	+++	0 81	0 0406	0 270	0 25	0.54
				0 0405		0 29	

diacetic urine, and also to detect definite changes in the intensity of the nitroprusside tests, which were always very intense (large purple ring) with the more concentrated solution just used. Exactly the same technique was followed for hydrolysis of the diacetic ester, but only about 1.5 gm. were used instead of 13 gm. After standing (slightly acidified because diacetic acid is more easily converted into acetone in an acid medium (6)) at room

temperature for 24 hours, the characteristic odor of the ester had disappeared. The solution was allowed to stand for about 48 hours and analyzed from time to time. The results are given in Table III.

The content of free acetone has nearly tripled but the amount of diacetic acid has decreased, in spite of the fact that the total ketone bodies have increased in concentration. After the 5th day of hydrolysis, an equilibrium seems to have been reached, and as hydrolysis has proceeded, the ferric chloride test has diminished, and the nitroprusside test increased, a phenomenon commonly observed in diabetic urine. For example see Table IV.

TABLE IV.

Time.	Ferric chloride test.	Nitroprusside test	Total acetone bodies Acetone + diacetic acid expressed as acetone (Van Slyke method).	Duplicates of weights of precipitates.	Free acetone (Folin method).	Duplicates of free ac- etone determination	Diacetic acid expressed in acetone. Difference of total acetone bodies and free acetone
			gm. per liter	gm	gm. per liter		gm.
Specimen of urine 15 min. after voided	+	+	0 25	0 0124 0 0123	0 11	0 10 0 12	0 14
Same specimen 24 hrs. later	Trace.	++	0 22	0 0112 0 0104	0 125	0 13 0 12	0 095

When the hydrolyzed solution of diacetic ester had reached the stage corresponding to the 3rd day of our chart (see Table III) a 100 cc. specimen was boiled under reflux condenser for 40 minutes. This on being tested, when sufficiently cool, showed negative qualitative tests. Quantitative determinations showed 0.24 gm. of diacetic acid per liter. Boiling had reduced the amount of diacetic acid to less than one-half, and it had also reduced the amount of acetone to about one-quarter of its original amount (notwithstanding the use of a reflux condenser), a result in direct disagreement with Harding and Ruttan's assumption that the acetone is increased after boiling. This decrease in the amount of acetone present may account in a large part for the disappearance of the sodium nitroprusside test.

The disappearance of diacetic acid such as occurred in Experiment 2 on page 351 when its solution is aerated and saturated with NaCl, is rather puzzling. Since it exists only in the form of its esters, the properties of this compound are unknown. The literature gives scant information on the subject. The first hypothesis which we considered was that diacetic acid might be volatile like acetone and carried off by the current of air. Therefore, we saturated with NaCl a mixture containing 0.315 gm. of acetone per liter and 2.63 gm. of diacetic acid, and aerated it through 10 cc. of 0.1 N NaOH and finally through iodine. The titration of the iodine solution showed that the concentration of free acetone was 0.298 gm. per liter and the 10 cc. of 0.1 N NaOH were exactly recovered. The amount of diacetic acid left was 1.23 gm. per liter. This experiment was repeated with the same result. It shows that diacetic acid is not carried off as such with the current of air, nor in form of a volatile acid into which it might possibly have been converted, in which case it would have neutralized part of the 0.1 N alkali. This procedure did not interfere with the quantitative determination of free acetone, which was all recovered except a trace. Nor can diacetic acid have been carried out in the form of acetone, because the free acetone quantitative determinations showed no increase.

We have aerated a pure solution of acetic acid through a known amount of alkali, for a period corresponding to those of our experiments, in order to test whether acetic acid could be carried over by the current of air in appreciable amounts. It showed very definitely to be the case. If, then, acetic acid could have been formed from diacetic acid during the aeration process, it would have been caught by the 0.1 N NaOH solution, which in the experiment above showed no change.

In order to ascertain whether NaCl,  $H_3PO_4$ , or kerosene had any effect on the diacetic acid, we performed the following experiment.

A solution, containing 2.10 gm. of diacetic acid and 0.40 gm. of acetone, was saturated with NaCl; to this were added 10 gm. of a 10 per cent solution of  $H_3PO_4$  and a little kerosene; the whole was left standing for  $\frac{1}{2}$  hour (with an occasional shaking). *This mixture was not aerated*; quantitative estimation showed that there remained 2.10 gm. of total acetone bodies in solution which corresponded to the initial diacetic acid, no free acetone



remained. From this it will be seen that the reagents added do not destroy diacetic acid. But subsequent aeration caused roughly one-half of the initial amount of diacetic acid to disappear. This is not carried off as diacetic acid nor acetic acid.

In order to prove that it does not enter into a new compound, the following experiment was carried out.

A sample of a solution of diacetic acid, acetone, and some ethyl diacetate containing 2.864 gm. per liter of total acetone bodies was saturated with NaCl,  $H_3PO_4$ , and kerosene were added in the usual way, and the mixture was aerated for  $1\frac{1}{2}$  hours. The total acetone bodies were now reduced to 0.945 gm. per liter. The solution was hydrolyzed for 24 hours in the presence of 0.7 per cent KOH. The concentration of total acetone bodies was again determined, and found unchanged.

Further information is needed to explain the disappearance of diacetic acid under the conditions of the experiment.

It became evident from our experiments, particularly those in which aeration was used, that the sensitiveness of the nitroprusside test was influenced by some of the substances used in the Folin free acetone determination, as well as by certain substances present in urine, whether normal or pathological. The following experiment was performed in order to determine the responsible agent.

Four test-tubes, each containing the same amount of a 1 per cent solution of acetone (see page 349), were treated respectively as follows:

To Tube I no additional substances were added

To Tube II a few drops of a 10 per cent solution of  $H_3PO_4$  were added.

To Tube III some kerosene was added and the tube shaken.

To Tube IV sufficient NaCl was added to saturate it.

Then the nitroprusside test was performed in the four tubes, which were subsequently allowed to stand for some time; the results are presented in Table V

It is obvious that whereas  $H_3PO_4$  had no effect at all on the reaction, kerosene had slight influence, while the NaCl had an immediate and very pronounced effect. The ring changed immediately into a heavy purple mass. The fluid at the bottom of Tube IV was pipetted out, retested with nitroprusside, and showed a ring with the same intensity. NaCl tested alone in solution with the nitroprusside reagent gave an absolutely negative result.

We finally diluted the 1 per cent solution of acetone with distilled water and with a 10 per cent solution of NaCl, and applied the nitroprusside test. Our results are given in Table VI.

When compared with the data of Table II, it becomes evident that NaCl intensifies the color, but does not produce a positive reaction at greater dilutions than those obtained with distilled water. If we remember that when the solvent is urine, the test stays positive until dilutions of 1 to 100,000 (by volume) it be-

TABLE V.

Time	Tube I	Tube II	Tube III	Tube IV.
Immediately after the test was performed ..	+	+	+	+++
$\frac{1}{2}$ hr. later ..	+	+	++	++++
$\frac{1}{2}$ " " .. ..	Trace.	Trace.	+++	++++
1 " " .. ...	0	0	+++	++++

TABLE VI.

Dilution	Solvent is water (see page 348).	The solvent is a 10 per cent solution of NaCl.
1 per cent (by volume)	+	+++
0.1 " " (" " )	Trace.	+
0.01 " " (" " )	0	0
0.001 " " (" " )	0	0

TABLE VII

*Influence of Various Salts on the Sensitiveness of the Nitroprusside Test.*

Test-tube	Control	I	II	III	IV	V
Salt	None	NaCl	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	MgSO <sub>4</sub>	CH <sub>3</sub> COOK	K oxalate.
Nitroprusside test	+	+++	+++	+++	+++	+++

comes clear that other substances than NaCl are present in urine, which increase the sensitiveness of the test or even produce the test.

As it was probable that the peculiar behavior of NaCl in regard to the nitroprusside test might be similar to the influence of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, which had been noticed long ago by Rothera, we felt

it would be of interest to test various neutral salts in the same way as sodium chloride and ammonium sulfate had been tested. Six test-tubes, each of 20 cc. capacity, containing, respectively, 4 to 5 gm. of various dry salts, were filled with a like amount (about 15 cc.) of the same specimen of diabetic urine. The nitroprusside test was performed on each specimen after time had been allowed for the various salts to dissolve. The intensity of the tests is given on Table VII.

It can easily be shown that the influence of the salt is proportional to its concentration, with a maximum effect at saturation.

*The Quantitative Significance of the Qualitative Tests.*

*Sodium Nitroprusside Test.*—Where the technique given on page 348 is followed, no electrolytes being used to intensify the color reaction, the following approximations have been made from the data of qualitative and quantitative findings on 260 specimens of diabetic urine. When the concentration of total acetone bodies expressed as acetone is less than 25 mg. per liter, the test is negative; from 25 to 50 mg. the test may be positive or negative. When it is positive, it is either "Trace" or "+." It is often negative for 50 mg. per liter and positive for 25 mg. per liter. The test is irregularly "Trace," "+," or "++" for concentrations, varying from 50 mg. to 0.5 gm. per liter.

For concentrations of 0.5 to several grams per liter, absolutely no rule can be ascertained. The test may be "+," "++," "+++," "++++," with no definite relation at all to concentration of total acetone bodies. This is evidently due to the fact that the proportion of diacetic acid plus acetone to the total acetone bodies is very variable, and also that many conditions, especially salt concentrations, influence the sensitiveness of the test.

*Ferric Chloride Test.*—Using the technique given on page 348 on the same specimens of diabetic urine, for concentrations of less than 0.10 gm. of total acetone bodies per liter, the test is always negative. It is irregularly positive (slightly) or negative for concentrations varying from 0.10 to 0.40 gm. per liter of total acetone bodies. It is constantly, but slightly, positive (brown-mahogany) from 0.4 to 1.0 gm. of total acetone bodies per liter. It is definitely positive from 1 to 2 gm. per liter, and for still higher concentrations, it becomes almost black.

## CONCLUSIONS.

1. A solution of pure acetone gives a color reaction with sodium nitroprusside.

2. A solution of diacetic acid, as free as possible from acetone, also gives a color reaction with sodium nitroprusside.

3. Such a solution of diacetic acid gives a color reaction with ferric chloride; acetone alone does not.

4. Electrolytes present in urine, especially NaCl, tend to intensify the color of the ring in the sodium nitroprusside test.

5. Quantitatively, because of the many interfering substances, the tests as routinely done serve as only crudest approximations in indicating the amounts of acetone and diacetic acid present. The ferric chloride test appears to give results somewhat less eccentric than the nitroprusside test.

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# THE EFFECT OF DIET ON THE CONTENT OF VITAMINE B IN THE LIVER.\*

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At the present time there seems to be a consensus of opinion that the food factors commonly known as vitamins A, B, and C are not synthesized in the organisms of the higher animals and that the latter are dependent upon exogenous food sources for the provision of their supply of essential vitamins. The probability of these assumptions is supported by considerable experimental evidence, particularly with respect to vitamins A and C. In a recent discussion of the subject Steenbock, Sell, and Nelson<sup>1</sup> reviewed the data indicating that the rat can store the fat-soluble vitamin in large amounts in its tissues for further use. In their own experiments they demonstrated how the content of rat livers in vitamin A may vary with the previous dietary history of the animals. The hepatic tissue of rats reared on rations low in fat-soluble vitamin and fed at a level of 1 per cent of the food mixture was insufficient as the sole source of vitamin A, whereas in otherwise comparable trials the same percentage of liver tissue from rats reared on rations of normal fat-soluble vitamin content sufficed.

There is also considerable accumulated evidence that the proportions of the familiar vitamins found in cow's milk vary in

\* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

<sup>1</sup> Steenbock, H., Sell, M. T., and Nelson, E. M., *J. Biol. Chem.*, 1923, lvi, 327.

correspondence with the fodder of the cattle in as far as the latter may have a widely varying content of vitamins derived from the plant sources employed.<sup>2</sup> The significance of this for the thesis under discussion is emphasized by the fact that the proportions of most of the chemical constituents of milk tend to remain fairly constant for a given breed despite wide variations in feed. This may be attributed to the circumstance that an organism tends to uniformity in the case of products that are manufactured *de novo* therein; to greater variability in the case of substances which are transported or eliminated in proportion to their supply to the body. The vitamins seem to belong to the latter group.

The question also arises as to how the supply of vitamine in the body may become depleted when there is a lack of it in the intake, and whether the different vitamins behave alike under these conditions. For example, is the vitamine "fixed" in the tissues, is it destroyed there in connection with the nutrient functions with which it may be involved, or is it lost to the body through the excretions? The answer to these inquiries may throw light upon the seemingly unlike needs of different species for the different vitamins. Thus the experience of investigators has given little, if any, convincing indication of the need for vitamine C in the rat if one may judge from the absence of symptoms of scurvy on a regimen upon which the guinea pig soon develops the disease. Parsons<sup>3</sup> has come to the conclusion that the apparent lack of susceptibility in the case of the rat is not explicable on the hypothesis that it does not need the antiscorbutic factor, but rather on the probability that the latter is retained in the rat organism even after feeding for a long period on a typical antiscorbutic diet. Other possibilities are, however, not yet exhausted.

Steenbock, Sell, and Jones<sup>4</sup> have become convinced that the rat has little ability to store vitamine B. They investigated the question by ascertaining whether the previous condition of feeding, with respect to the vitamine B content of the ration, affected the capacity of young animals to survive on a vitamine-free regimen. We

<sup>2</sup> The subject is discussed by Kennedy, C., and Dutcher, R. A., *J. Biol. Chem.*, 1922, 1, 339.

<sup>3</sup> Parsons, H. T., *J. Biol. Chem.*, 1920, xliv, 587.

<sup>4</sup> Steenbock, H., Sell, M. T., and Jones, J. H., *J. Biol. Chem.*, 1923, lv, 411.

have attempted to learn whether a typical storage tissue—the liver of the rat—shows variations in its content of vitamine B depending on the richness of the diet in this factor. The organs were removed from rats that (1) had lost weight and shown severe nutritive decline on a diet adequate except for the lack of vitamine B; or (2) had been kept on a wholesome mixed diet that promoted health and vigor. That normal liver tissue contains a liberal content of vitamine B has already been established.<sup>5</sup>

The fresh livers were washed free from superficial blood, then comminuted in a small mortar, spread on enamelled pans, and dried in a blast of air at about 50°C. The resulting product was ground fine and compressed into small tablets of the weight desired.

The tests were made by noting the effects of feeding the dried liver tissues to young rats which were failing to grow on a diet lacking vitamine B. The standard unsupplemented food mixture consisted of

	<i>per cent</i>
Casein . . . . .	18
Starch . . . . .	54
Butter fat . . . . .	9
Lard . . . . .	15
Salt mixture <sup>6</sup> . . . . .	4

The liver tissue supplements to be tested as possible sources of vitamine B were fed apart from this standard mixture in doses ranging from 50 to 200 mg. per day. The outcome of these "curative" tests is best indicated in Chart 1.

In every instance in the case of the liver from the well fed animals a favorable response was obtained—sometimes with as little as 50 mg. per day of the dried tissue (Rat 8425). With 100 mg. growth at almost normal rate was secured, and with doses of 150 to 200 mg. the effect was even more marked (Rats 8393, 8394, and 8429). Liver thus appears to be almost as good a source of vitamine B as is an equal amount of yeast (Rats 8392 and 8436).

The results following the use of livers from rats deprived of vitamine B are in strong contrast with the foregoing. Even with

<sup>5</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309; 1918, xxxiv, 17.

<sup>6</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.



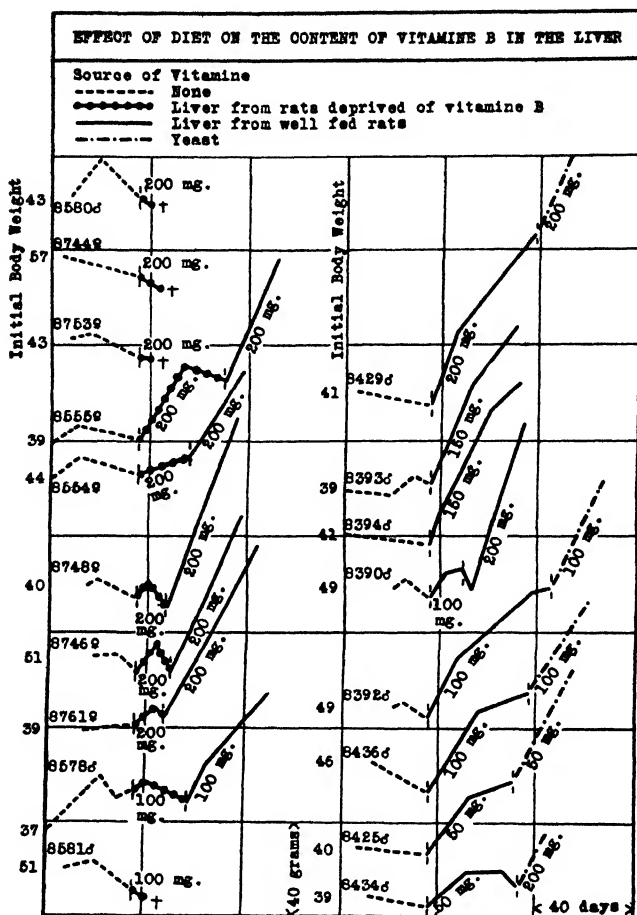


CHART 1. Showing the effect of comparable daily doses of dried rat liver as sources of vitamine B upon the rate of growth of rats receiving an otherwise adequate food mixture as described in the text. During Period 1, no liver tissue whatever was supplied, so that all the animals failed to continue their growth. Beginning with Period 2, the vitamine B supplements were derived from the livers of animals respectively declining on diet lacking vitamine B (indicated by ●●●●●), or thriving on a full mixed ration (indicated by ———). The figures on the graphs indicate the daily dosage of dried liver tissue. In some cases the animals were further tested, for the sake of comparison, by replacing the liver tissue with an equal weight of dried brewery yeast (for which many data have been reported by us (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1922, liv, 739)).

the highest dosage—200 mg.—the outcome was unsuccessful, the rats either failing to make adequate gains or declining and dying (Rats 8580, 8753, 8744, 8746, 8748, and 8761). The contrast in the same animal between the effect of the same daily dose of liver from animals deprived of or adequately supplied with vitamine B is striking (Rats 8554, 8555, 8578, 8746, 8748, and 8761).

From the clear-cut outcome of this study the conclusion seems inevitable that *when an adequate supply of vitamine B is lacking in the diet the store of this factor in the liver tissue, where it is ordinarily found in abundance, becomes largely depleted.*



# DIGESTION EXPERIMENTS WITH THE RAW WHITE OF EGG.

## II. THE DIGESTIBILITY OF UNBEATEN IN COMPARISON WITH BEATEN WHITES.

By MARY SWARTZ ROSE AND GRACE MACLEOD.

(From the Department of Nutrition, Teachers College, Columbia University, New York.)

(Received for publication, October 13, 1923.)

In a previous communication<sup>1</sup> experiments were reported on human subjects taking from ten to twelve raw egg whites per day as a part of a simple mixed diet. The average coefficient of digestibility for the protein of the whole ration was 82 per cent, from which that for the egg whites alone was estimated to be 80 per cent. The same ration with the eggs cooked showed a coefficient of 86 per cent for the protein of the whole ration, estimated as 82 per cent for the eggs alone.

In these experiments there appeared to be better utilization of the raw whites when finely beaten than when taken with little subdivision. This point has been subjected to further investigation with the result that the differences between the two are less marked than they at first appeared.

Six young women took the same ration as in the former experiment<sup>2</sup> for two 3 day periods. In the first period the egg whites

<sup>1</sup> Rose, M. S., and MacLeod, G., *J. Biol. Chem.*, 1922, 1, 83.

<sup>2</sup> Protein content of the ration of this series of experiments:

Food material	Daily intake	Protein (N $\times$ 6.25).
	gm.	gm.
Egg white .....	375	39.54
Rice .....	85	6.56
Saltines .....	35	3.35
Lettuce .....	85	1.15
Fruit juice .....	600	3.22
Cream .....	79	1.51
Butter .....	39	0.38
Olive oil*.....		
Sugar.....	50	

\* Taken by only one of the group (33 gm. per day).

were subdivided as little as possible; in the second they were very thoroughly beaten. The data are given in Table I.

TABLE I.

*Coefficients of Digestibility for Raw Egg Whites (Beaten and Unbeaten).*

Subject	Period I (egg whites beaten)			Period II (egg whites unbeaten).		
	N in feces × 6 25	Coefficients of digestibility.		N in feces × 6 25	Coefficients of digestibility.	
		Whole ration.	Egg white only *		Whole ration	Egg white only.*
	gm	per cent	per cent	gm	per cent	per cent
G. C.	5 13	90 8	92.7	7 66	86 2	86 2
A. E.	7 95	85 7	85 6	7 96	85 7	85 6
H. F.	10 55	81 1	79 0	9 81	82 4	80 9
D. H.	5 34	90 4	92 2	11 40	79 3	76 9
E. H.	4 87	91 2	93.4	7 02	87 4	88 0
G. S.	7 63	86 3	86.4	6 11	89 0	90 3
Average.		87 6	88 2		85 0	84 7

\* Estimated by making the following allowances for loss in digestion of the other foods in the diet.

	per cent
Rice and lettuce	. . 17
Fruit juice	. . . 15
Saltines	. . . 12
Cream and butter	. . . 3

TABLE II

*Differences between Coefficients of Digestibility of Beaten and Unbeaten Raw Whites of Eggs in Favor of Beaten Whites.*

Subject	Period I For whole ration	Period II For egg whites only (estimated)
	per cent	per cent
G. C	+4 6	+6 4
A. E	0 0	0 0
H. F.	-1 3	-1 9
D. H.	+11 1	+15 3
E. H	+3 8	+5 4
G. S.	-2 7	-3 9
Average	+2 6	+3 6

In one case there was no apparent difference in the utilization of the unbeaten as compared with the beaten whites; in two cases the unbeaten whites showed a higher coefficient than the

beaten; in only one case was there a markedly better utilization of the beaten whites. Computed for the ration as a whole, the average difference in the coefficients of digestibility was 2.6 per cent, in favor of the beaten whites; for the whites alone, 3.6 per cent, the returns of the individual cases being as shown in Table II.

## SUMMARY.

Finely subdivided raw egg whites tend to be utilized somewhat more completely than those taken without any subdivision whatever, though one-half the cases showed as good digestion of the unbeaten as of the beaten whites, if not better. In only one case was there a marked difference in favor of the beaten whites.

In twenty-two experiments with raw white of egg, the average coefficient of digestibility for the protein of the whole ration is 84 per cent (estimated as 83 per cent for egg white only); in

TABLE III  
*Range of Coefficients of Digestibility of Protein of Whole Ration.*

Egg white	No of cases	Range of coefficients of digestibility.
		<i>per cent</i>
Cooked	10	81 8-90 6
Raw (all cases)	22	75 7-91.2
" beaten	9	81 4-90 8
" unbeaten	9	75 7-89 0

ten experiments with cooked whites, 86 per cent for both ration as a whole and cooked whites only. The range in the different experiments is shown in Table III.

It will be readily seen that the range for cooked whites and for raw beaten whites is practically the same; while for raw unbeaten whites it is somewhat lower.

We take this occasion to acknowledge the criticism of Wolf and Osterberg<sup>3</sup> that they were quoted incorrectly in our former paper. Owing to the error of a copyist, which we greatly regret, they were reported as having had two experimental periods on raw white of egg whereas they actually had only one, in which the loss of nitrogen in the feces was 41 per cent.

<sup>3</sup> Wolf, C. G. L., *J. Biol. Chem.*, 1922, lii, 207.



# A NEW SULFUR-CONTAINING AMINO-ACID ISOLATED FROM THE HYDROLYTIC PRODUCTS OF PROTEIN.

## II. SULFUR EXCRETION AFTER INGESTION.

By J. HOWARD MUELLER.

(From the Department of Bacteriology, Harvard Medical School, Boston.)

(Received for publication, October 11, 1923.)

An amino-acid, which apparently has the formula  $C_6H_{11}SNO_2$ , has recently been isolated by the writer from the hydrolytic products of several proteins, and described.<sup>1</sup> Considerable evidence was adduced tending to show the substance to be a primary cleavage product of protein, but, pending the determination of its structure and a further study of its properties, the question was considered to be unsettled. In order to throw additional light on the source of the compound, a few experiments have been carried out in which, after the ingestion of 0.5 to 1.0 gm. of the material, the urinary sulfur has been determined.

It is evident from Tables I to V that the sulfur is easily split out of the compound and oxidized by the body to the form of inorganic sulfate, since in its original form it would, of course, appear in the results as unoxidized or neutral sulfur. The single experiment in which nitrogen was determined indicates that the results cannot be explained as a possible stimulation of metabolism with a breaking down of sufficient body protein to account for the extra sulfur, for in that case the nitrogen excretion should have been almost doubled.

Had the increase appeared in the neutral sulfur fraction, indicating the excretion of the compound in an unchanged condition, it might have indicated that one was dealing with a compound foreign to the body and, therefore, not a primary cleavage product of the protein molecule. However, since oxidation of other than naturally occurring substances may often be affected in the body,

<sup>1</sup> Mueller, J. H., *J. Biol. Chem.*, 1923, lvi, 157.



the results merely strengthen the other evidence, mostly indirect, already presented tending to show that the compound may be a primary product of protein digestion.

TABLE I.  
*Experiment 1.*

Control.

Period	Sulfur in period.		
	Inorganic SO <sub>4</sub>	Ethereal SO <sub>4</sub>	Neutral S
	<i>mg</i>	<i>mg</i>	<i>mg.</i>
1	19 8	0 6	5 4
2	19 1	2 1	6 0
3	15 9	2 3	5 0
4	15 7	2 1	5 5
5	10 6	2 0	3 5

TABLE II  
*Experiment 2.*

Control.

Period	Sulfur in period		
	Inorganic SO <sub>4</sub>	Ethereal SO <sub>4</sub>	Neutral S
	<i>mg</i>	<i>mg</i>	<i>mg.</i>
1	16 5	1 5	4 8
2	19 3	1 8	4 9
3	16 5	2 0	5 7
4	16 6	2 3	6 5
5	16 6	2 8	8 0

TABLE III  
*Experiment 3.*

0.5 gm of C<sub>6</sub>H<sub>11</sub>SNO<sub>2</sub> taken at end of third period.

Period	Sulfur in period		
	Inorganic SO <sub>4</sub>	Ethereal SO <sub>4</sub>	Neutral S
	<i>mg</i>	<i>mg</i>	<i>mg.</i>
1	17 0	1 5	7 7
2	13 2	1 8	6 9
3	17 2	2 4	6 3
4	24 6	0 5	6 4
5	22 7	0 9	6 5

#### EXPERIMENTAL.

The urine was collected in hourly periods beginning in the morning without eating breakfast, and no food was taken during the

course of the experiment. After collecting two or more specimens to determine the normal rate of excretion, the amino-acid was swallowed, dissolved in a little water, and the collection of specimens continued for several hours. The three forms of urinary sulfur were determined by the benzidine method of Fiske<sup>2</sup> and

TABLE IV.  
*Experiment 4.*

1.0 gm. of  $C_6H_{11}SNO_2$  taken at end of fourth period.

Period	Sulfur in period.		
	Inorganic $SO_4$	Ethereal $SO_4$	Neutral S
	mg	mg.	mg
1	15 2	1 1	4 1
2	15 5	1 1	3 6
3	13 9	1 3	3 8
4	14 5	2 2	4 9
5	17 3	1 7	4 8
6	28 7	1 3	5 4
7	30 5	1 4	5 2
8	30 3	1.5	4 4

TABLE V  
*Experiment 5.*

1.0 gm. of  $C_6H_{11}SNO_2$  taken at end of second period.

Period	Sulfur in period.			Nitrogen in period.
	Inorganic $SO_4$	Ethereal $SO_4$	Neutral S	
	mg	mg	mg.	gm
1	23 4	1 1	5 6	0 57
2	19 5	1 4	5 5	0 52
3	25 0	1 5	7 4	0 55
4	31 4	2 0	6 1	0 48
5	39 4	0 7	7 3	0 56
6	41 4	1 5	5 4	0 50
7	34 6	1 7	6 5	0 54
8	34 2	2 0	6 9	0 49

the total amounts of the different types calculated for the period. In one experiment total nitrogen was also determined by the Kjeldahl method. Two experiments are included, in which no sulfur compound was taken, to serve as controls on the others.

The results are presented in Tables I to V.

<sup>2</sup> Fiske, C. H., *J. Biol. Chem.*, 1921, xlvii, 59.



## NOTES ON THE ETHER EXTRACT OF FECES.

By ARTHUR D. HOLMES

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AND ROBERT H. KERR.

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(Received for publication, October 1, 1923 )

In connection with studies of the digestibility of some 50 edible fats carried on in the Office of Home Economics of the United States Department of Agriculture, two distinct but closely related questions have arisen regarding the nature of the ether extract of feces. One is whether the usual method of extracting feces with ether involves any serious error because of the presence of fatty soaps; the other is, how the fat of the diet compares in nature with the ether extract of the feces, or as it is ordinarily called, the fecal fat. The present paper brings together data bearing on these two questions.

### *Methods of Determining Fat in Feces.*

It has been generally believed that in the metabolism of fats a portion of the unutilized fat is excreted in the form of soaps. The amount of unutilized fat thus excreted has not been considered large enough to be significant, and the common method for determining fat in feces by extraction with ether does not make any allowance for such fatty soaps, which are little affected by this solvent.

In considering methods to be followed in the Office of Home Economics, it became necessary to ascertain the extent of the error involved by disregarding the soaps; therefore, the ether extraction method was supplemented by one designed to recover the soaps as well as the ether-soluble fat. Of various procedures, the one

proposed by Folin and Wentworth<sup>1</sup> is perhaps the best known, and was adopted in these tests. It consists essentially in extracting the sample of air-dried feces for 20 hours with an ethereal hydrochloric acid solution containing 10 per cent acid, and in further extracting with petroleum ether the fatty material remaining after evaporation.

Five fats were studied and the coefficients of digestibility obtained are given in Table I.

TABLE I.

*Coefficients of Digestibility Obtained by Two Methods of Determining Fat in Feces.*

Laboratory No	Subject	Fat studied	Coefficients of digestibility.	
			Ether extraction method	Folin-Wentworth method
			<i>per cent</i>	<i>per cent</i>
56	E. S. M.	Lard	94.5	95.3
86	I. B. B.	Butter.	95.3	95.8
264	R. L. S.	Goose fat.	96.5	97.0
275	D. G. S.	Chicken fat	93.5	94.1
305	P. E. M.	Egg yolk fat.	92.1	92.6

It will be noted that the coefficients obtained by the ether extraction method are slightly, but consistently, lower than those obtained by the Folin-Wentworth method. This is contrary to what would be expected if any appreciable amount of soap were present in the feces. However, the differences in the results obtained by the two methods are less than those obtained from feces of a single subject eating the same diet during different test periods.

#### *Nature of Ether Extract of Feces.*

The ether-free residue obtained from the ether extract of the feces was ordinarily very dark brown or black, very viscous, and, in some instances, solid at room temperature. The odor was pronounced and with one exception uniform. In the case of diets in which relatively large quantities of cocoa butter were consumed, the resulting feces and the ether extract of the feces

<sup>1</sup> Folin, O., and Wentworth, A. H., *J. Biol. Chem.*, 1909-10, vii, 421.

had a pronounced pleasant odor of chocolate fat. In general, however, regardless of the subject, the kind of edible fat under consideration, or the method of extraction, the material extracted, as far as could be judged from physical appearance, seemed to be uniform. The work of Hill and Bloor<sup>2</sup> also indicates a continuous output in the feces of "fat," the composition of which is constant and independent of the diet.

### *Experiments with Fecal Fat.*

In order to obtain more information concerning the nature of the so called fecal fat, examination was made of the ether extract from feces resulting from tests with three typical fats of high digestibility, goose fat and oleo oil for animal and corn oil for vegetable fat. The feces, furnished by the several subjects in the experiments on each fat, were carefully mixed before extraction, so that any individual peculiarities would be offset as far as possible.

It was noted that the dark, viscous residue from the ether-extracted material obtained from feces resulting from these fats was not completely redissolved in petroleum ether. The conclusion drawn was that the ether had extracted from the feces some substance, or substances, which would not have been extracted by the petroleum ether. Accordingly, similar samples were extracted with petroleum ether under identical conditions, the petroleum ether was volatilized, and the residue placed in contact with fresh petroleum ether. In this case also, it was found impossible to redissolve the entire residue in petroleum ether, although it had previously been in solution, or suspension, and had, at any rate, passed through filter paper, or alundum extraction filters, or both.

Table II summarizes data obtained from the tests reported in this paper.

A comparison of the iodine number and the saponification value of the fecal fat with the iodine number and saponification value of the natural fat shows the ether extract of the feces to be a quite different substance from the fat that was ingested.

<sup>2</sup> Hill, E., and Bloor, W. R., *J. Biol. Chem.*, 1922, liii, 171.

An examination of the ethereal extract of the feces from the oleo oil digestion experiments gave the results which follow:

	per cent
Total unsaponifiable matter . . . . .	42 80
Insoluble in alcohol or in ether . . . . .	24 18
Soluble in alcohol and in ether . . . . .	18 62
Fatty acids . . . . .	53 45

The portion of unsaponifiable matter insoluble in alcohol was dry, powdery, odorless, and of a light brown color. It did not melt when heated, but burned quietly and without flame, first to a char, and finally to a clean white ash. This material thus

TABLE II  
*Results of Chemical Examination of Fecal Fat.*

Kind of fat	No of experiments	Coefficients of digestibility	Chemical characteristics.			
			Natural fat		Fecal fat	
			Iodine number	Saponification value	Iodine number	Saponification value
		per cent				
Goose fat	7*	95 2	68 53	194 76	28 57	159 11
Oleo oil.	8†	98 8	44 31	207 27	27 93	146 17
Corn "	7‡	96 9	123 10		34 26	108 60

\* Langworthy, C. F , and Holmes, A. D , *U S Dept. Agric., Bull 507*, 1917, 6

† Holmes, A. D , *U S Dept. Agric , Bull 613*, 1919, 12.

‡ Holmes, A. D., *U. S. Dept. Agric., Bull 687*, 1918, 3.

appeared to be not fat, but a portion of the solid constituent of the feces, mechanically carried through the filter in finely divided condition. The percentage of unsaponifiable material soluble in alcohol, which is many times greater than is normal to oleo oil, may be accounted for by the presence of coprosterol and cholesterol, which are known to be normal constituents of feces.

The iodine and saponification numbers also show the composition of the extract to be widely different from that of the diet fat. These constants are, it is true, affected to a large degree by the presence of the unsaponifiable matter. That this does not wholly account for the difference is shown by the iodine number of the

fatty acids, which was found to be 35.18 instead of approximately 50 as it should have been for an oleo oil of iodine number 44.

Referring to the tests with corn oil, it has been assumed that the 16.54 per cent of the ether extract of the feces, which does not appear as unsaponifiable matter soluble in ether or fatty acids, consists principally of the undissolved solid matter similar to that referred to in connection with the experiments with oleo oil.

Examination of the ether-soluble unsaponifiable matter showed it to consist largely of coprosterol. This was identified by crystallization from 80 per cent alcohol. Characteristic needle-shaped crystals melting at 94.8°C. were obtained. There was also present another body, evidently either cholesterol or sitosterol, but not in sufficient amount to permit identification.





## FAT-SOLUBLE VITAMINS.

### XVI. STABILITY OF THE ANTIRACHITIC VITAMIN TO SAPONIFICATION.\*

BY H. STEENBOCK, J. H. JONES, AND E. B. HART.

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison )

(Received for publication, October 22, 1923.)

Three years ago Steenbock, Sell, Nelson, and Buell presented data before the American Society of Biological Chemists showing that vitamin A, then spoken of as the fat-soluble vitamin, was resistant to drastic saponification as carried out by boiling with 20 per cent alcoholic potassium hydroxide (1). With the recognition that rickets can be prevented by a substance which, like vitamin A, is soluble in fats, the most obvious thing to do was to determine if this principle was likewise stable to such treatment. These experiments were carried out in the spring of 1922 with the particular object in mind of ascertaining if the antirachitic property like the antiophthalmic property was resident in the same or in different compounds. It was found (2) in confirmation of experiments by Zucker, Pappenheimer, and Barnett (3), whose efforts in this direction were entirely unknown to us, that the antirachitic vitamin is very resistant to destruction by strong alkali. In the meantime it has been shown by McCollum, Simmonds, Becker, and Shipley (4), Steenbock and Nelson (5), and Steenbock, Hart, Jones, and Black (6) that vitamin A is distinct from the antirachitic vitamin. In this light the presentation of our results in detail in this paper may appear gratuitous were it not for the fact that we anticipate that there are those who may still be skeptical of the conclusiveness of the evidence after reading the preliminary papers by Zucker and ourselves.

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

## EXPERIMENTAL.

Our results were obtained by experiments on dogs. As a preliminary, experiments were also carried out with rats to determine to what extent saponified cod liver oil is equivalent to the unsaponified material in supporting growth, because obviously it was necessary to have some measure of the comparative value of the two to determine the amount to be administered.

That vitamin A is stable to mild treatment with weak alkali in the cold was shown by McCollum and Davis (7). They took butter fat in alcohol-petroleum-ether solution and allowed it to stand mixed with a solution of alcoholic potassium hydroxide until "saponification was complete." The soaps were then freed from solvent, taken up in water, and the unsaponified materials taken up in ether-olive oil solution. The olive oil freed from ether was found efficient as a source of vitamin A. Unfortunately, McCollum and Davis did not make a test to determine if saponification was complete other than merely by the appearance of the mixture. Steenbock, Sell, and Buell (8), and later Steenbock, Nelson, and Hart (9) showed that very drastic treatment with strong alkali in the hot does not destroy it, even though the fats are completely saponified. The saponified cod liver oil used in the present experiments was prepared by the former method of saponification: 500 gm. of cod liver oil were boiled in two portions, each with 500 cc. of 20 per cent alcoholic potassium hydroxide for 30 minutes. After cooling and diluting with 3,500 cc. of water they were extracted thrice with generous portions of ether. The extracts were united, brought down to dryness, taken up with 200 cc. of alcohol, 50 cc. of 20 per cent alcoholic potash added (giving a concentration of approximately 4 per cent potassium hydroxide), and the solution was again boiled for 30 minutes to insure complete saponification. At the end of this time the solution was again diluted with water and thoroughly extracted with ether. After distilling off most of the ether the remainder, carrying the unsaponifiable constituents, was evaporated on the rations.

The results of feeding this material to rats for comparison with the unsaponified oil are presented in Chart I. Quantitatively, the curves of growth indicate that the saponified cod liver oil had not lost any appreciable amounts of its growth-promoting properties in the process of saponification and extraction employed.

This indicated two things. It showed, in the first place, that the vitamin A content had not been sacrificed, otherwise growth would have ceased, due to an insufficiency of this dietary constituent as indicated by ophthalmia, and, in the second place, it gave indirect evidence that the antirachitic vitamin had not been destroyed (5).

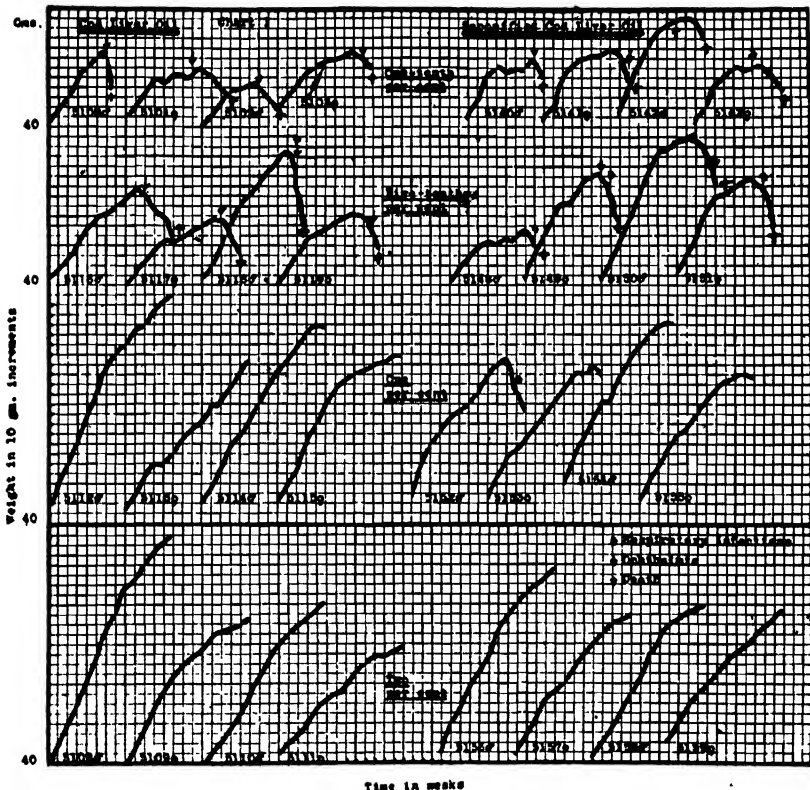


CHART I. Effect of saponification. Shows the comparative amount of growth obtained with cod liver oil or its equivalent of the ether extract of the saponified cod liver oil. At a level of 0.1 per cent all the rats developed ophthalmias. At 0.5 per cent all except 2, viz. Rats 5149 and 5150, were afflicted with ophthalmia and these contracted infections of the respiratory tract which is likewise to be taken as evidence of a lack of sufficiency of vitamin A. At the 1 and 2 per cent levels all the rats except Rat 5152 remained normal, it contracted pulmonary infections.

The indirect evidence in regard to the persistence of the anti-rachitic vitamin that we refer to is the fact, which has been generally neglected, that without the presence of the antirachitic vitamin prolonged growth of the experimental animals, as raised in our stock and kept in our laboratory, is impossible. Chart II shows the amount of growth possible on the basal ration of alcohol-extracted casein 18, salts 32, 4, agar 2, yeast 2, and dextrin 74. Though incidence of ophthalmia was rather long deferred in these animals the amount of growth was negligible due to lack of the antirachitic vitamin as we have shown in a previous paper where animals were fed on the same ration (5). These preliminary data on rats, interpreted in the light of our other experiments, gave us every reason to believe that our final experiments with dogs would show the vitamin to be entirely stable to saponification.

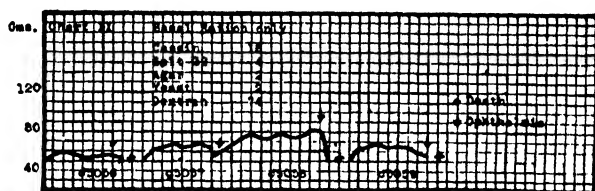


CHART II. Illustrates the early cessation of growth but delayed incidence of ophthalmia resulting from maintenance of rats on the basal ration fed to the animals of Chart I. This early failure of growth is due to an insufficiency in the amount of the antirachitic vitamin stored in the body relative to the animal's needs.

Five pups from one litter of mixed bull dog-beagle parentage were placed on our standard basal ration (6) of equal parts of rolled oats and white corn meal autoclaved at 15 pounds for 1 hour with a daily portion of 5 gm. of casein, 2 gm. of sodium chloride, 5 gm. of dicalcium phosphate, and 200 cc. of skimmed milk autoclaved at 15 pounds for 1 hour. Two of the pups received in addition the ether extract of 5 gm. of saponified cod liver oil and one pup 5 cc. of untreated cod liver oil.

The two animals kept on the basal ration without supplement soon stopped growing as shown in Chart III. After 4 weeks on the basal ration they experienced some difficulty in maintaining their balance; at 6 weeks their legs were markedly deformed and

walking was a matter of difficulty. Spasms also were occasionally observed. Where the basal ration was on the other hand supplemented with cod liver oil, saponified or untreated, growth was very uniform and continued so till the experiment was terminated.

Fig. 1 shows the marked contrast in the appearance of the animals 9½ weeks after the experiment was begun. Especially noticeable is the tendency of Dog 35 to "sprawl," due to inability on its part to support its body weight. In general we have noticed

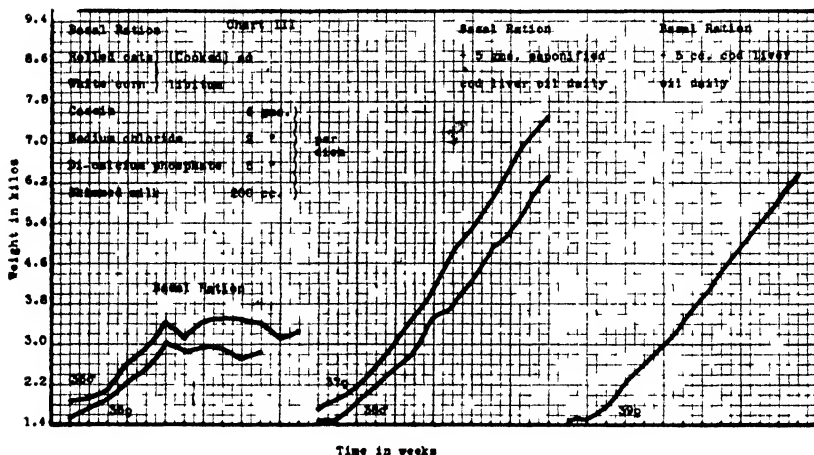


CHART III. Comparative growth of dogs. With the failure of growth of the dogs on the basal ration various symptoms were observed. Dog 35 on being awakened in the morning always appeared dizzy after the 27th day. By the 56th day it was markedly deformed and walked with considerable difficulty. On the 67th day it was observed in tetanic spasms. Dog 36 showed the same signs of dizziness on the 28th day. Fore legs were distinctly bowed and spread apart. By the 39th day it was unable to stand but later it was slightly improved.

that animals afflicted with rickets do not appear to be grossly distressed or in pain until late in the course of the disease. It is usually not until then that failure of appetite results and emaciation ensues. This maintenance of weight with growth in size and gradual decalcification of the skeleton leads to great strain being put upon the skeleton which often results in marked deformation.

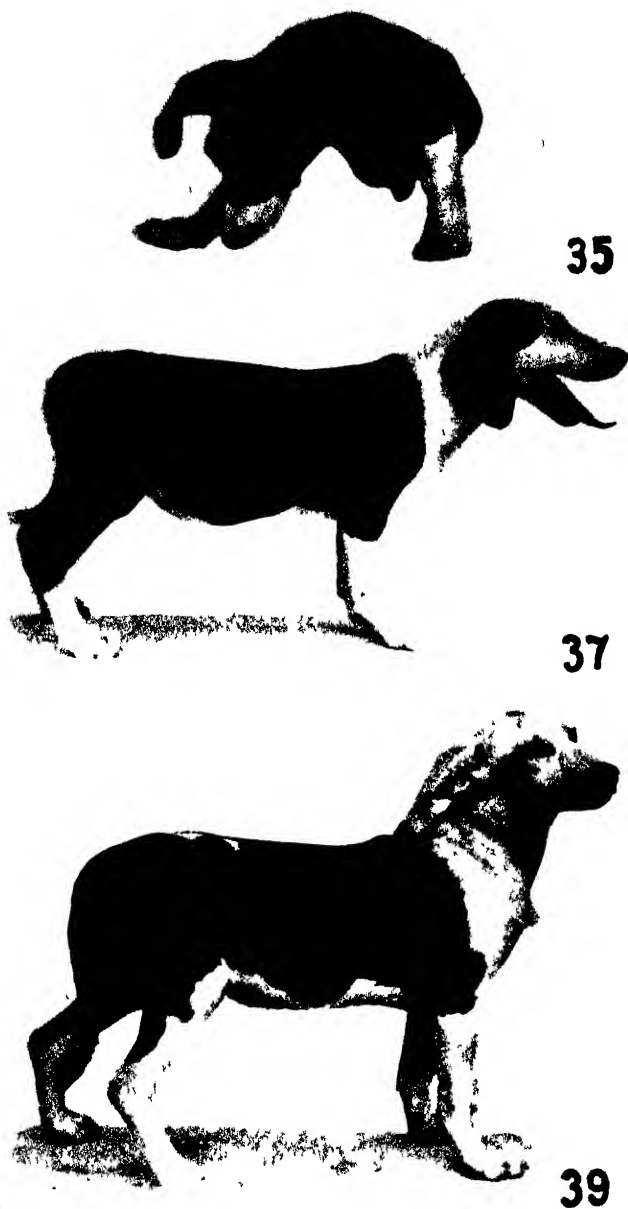


FIG. 1. Photographs of dogs 9½ weeks after the beginning of the experiment. Note the "flat footedness" and the curvature of the left fore leg of Dog 35.

This is brought out, though in a rather limited way, in Fig. 2 which shows sections taken through the thorax to include the fifth rib at the end of the experiment. The sections of Dogs 35 and 36 show not only a number of partly repaired fractures but also very much enlarged costochondral junctures. Dogs 37 and 38, like Dog 39, showed no fractures and much smaller costochondral junctures.

Anatomically, Fig. 3 presents decided evidence in the form of radiographs for the equivalency of the saponified cod liver



FIG 2 Thoracic arches showing the fifth ribs with fractures and enlarged costochondral junctures in Dogs 35 and 36

oil with the untreated oil for promoting normal calcification. These were taken 11 weeks after the experiment was started. While Dog 36 on the basal ration showed very poor calcification, Dogs 37 and 39 both showed excellent calcification.

Evidence obtained by x-ray examination in regard to deficient deposition of the inorganic elements in bone was supported by analyses for ash. For these analyses the femur of the right leg was dissected out from the carcass at death, dried at 96°C. for a week, and after crushing in a screw-press was thoroughly extracted, first with alcohol and then with ether in a Caldwell extractor. The residue was thoroughly dried, then weighed, and ashed in an





FIG 3. Radiographs of hind legs showing abnormal bone structure and deficient calcification of the epiphyses in Dog 36.

electric furnace. Percentage of ash was calculated on the basis of the ether-alcohol-extracted bone.

Table I presents the data on these ash analyses. As there recorded, the dogs receiving cod liver oil either in the form of the unsaponifiable ether-soluble material or as untreated oil show a rather uniform ash content in the neighborhood of 45 to 47 per cent. The basal animals depart materially from these values, their femurs contained only 28 per cent of ash. This difference was clearly noticeable before ashing; the bones of the basal animals were very light and so fragile that adhering periosteum and connective tissue were removed with difficulty after they had been dried. Table I also shows that the bones of the different animals did not vary appreciably in diameter, but growth in length

TABLE I.  
*Comparison of Femurs.*

Dog No	Ration.	Length	Short diameter	Long diameter.	Ash	
		cm	cm	cm	gm	per cent
35	Basal.	8 10	1 00	1 10	1 69	28 28
36	"	8 55	1.05	1 20	1 91	28 86
37	Saponified cod liver oil.	11 00	1 00	1 15	6 27	46.45
38	" " " "	11 10	0 95	1 10	5 46	45 59
39	Cod liver oil.	11 10	0 95	1 00	5 75	47 61

had been markedly interfered with. This brings out the fact that the difference in the weight of the animals shown in Chart III was not alone due to emaciation—which ultimately was distinctly evident in the basal group—but that growth itself had been actually interfered with.

Table II presents data on another criterion introduced largely by Howland and Kramer (10) in the diagnosis of rickets. They showed that in severe rickets the inorganic phosphorus of the blood serum is much reduced in amount while the calcium in the same may or may not be affected. We have discussed these relations in another publication (6), pointing out the fact that rickets may be prevalent without impairment of the normal Ca and P content yet it appears that with increase in severity of the disease sooner or later the amounts in the blood stream, especially with relation to

phosphorus, are decreased. Table II shows a lower content of P in Dogs 35 and 36 on the basal ration than either Dogs 37 or 38 getting the saponified cod liver oil or Dog 39 getting the untreated oil. While the latter two groups had higher values 3 weeks later, the basal animals showed a decrease to less than one-half of the original figures. These determinations were all made by the Marriott and Haessler technique (11).

The calcium determinations were made by the Kramer and Tisdall method (12). As shown in the table the saponified cod liver oil was equal in value to the untreated oil for maintaining normal values; while the basal animals had from 7 to 8 mg. of calcium in their sera, the others ranged from 10 to 13 mg. It is

TABLE II  
*Composition of Blood per 100 Cc. of Serum.*

Dog No	Ration	Calcium *		Phosphorus †	
		6 wks on ration	9 wks on ration	6 wks on ration	9 wks on ration
		mg	mg	mg	mg
35	Basal.	7 59	7 99	4 96	2 23
36	"	7 99	7 32	4 60	1 67
37	Saponified cod liver oil.	11 32	11 72	7 88	8 57
38	" " " "	10 87	12 98	7 67	9 75
39	Cod liver oil.	11 12	12 25	7 07	9 00

\* Kramer and Tisdall.

† Marriott and Haessler.

difficult to say what significance is to be attached to the lowered Ca values. Both of the animals were observed at intervals in severe tetanic convulsions, but we have also observed this in dogs afflicted with rickets which had a normal calcium content of the blood. Apparently, a decreased calcium content cannot *per se* be the cause of the convulsions in these animals.

#### SUMMARY.

It is shown that, from the standpoint of growth, calcification of bones, and maintenance of normal calcium and phosphorus content of the blood, the ether extract of saponified cod liver oil is as efficacious as the untreated oil.

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# MOLECULAR CONFIGURATION IN THE SUGARS AND ACID PRODUCTION BY *BACILLUS GRANULOBACTER* *PECTINOVORUM*.

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In a previous communication from this laboratory Robinson (1) has shown that it is possible to ferment a number of sugars, which differ very markedly in their molecular configuration, by means of an organism which produces acetone and butyl alcohol. The fermentations may be divided into two groups. The first group, said to be normal, contains those in which the carbohydrate is almost if not completely utilized. Such fermentations are also characterized by the manner in which the titratable acidity of the medium rises during the first period of the fermentation, and falls at about the same rate during the second. On the other hand, the abnormal fermentations are characterized by a low consumption of carbohydrate, poor yields of neutral products, and a titratable acidity curve which remains at or near the maximum during the second half of the fermentation. It was found that the carbohydrates giving rise to such fermentations, namely xylose, arabinose, galactose, mannitol, melezitose, and inulin, do not belong to any one of the larger groups, and that there is no apparent structural relationship between them.

The present investigation was undertaken with a view to the further elucidation of the nature and rates of formation of the different acids produced during a normal fermentation, and to discover if possible, by a more detailed examination of the abnormal fermentations, whether there is in reality a direct relationship between the structure of the carbohydrate utilized and the products of the fermentation.

## EXPERIMENTAL METHODS.

The carbohydrates investigated were as follows: starch, in the form of maize meal, and glucose giving normal fermentations; and arabinose, xylose, galactose, and mannitol giving abnormal fermentations. The alcohol dulcitol was found to be unfermentable by this organism. These sugars and alcohols were obtained from the Special Chemicals Company, and were used without recrystallization.

*Medium.*—The synthetic medium used for the sugar fermentations contained:

K <sub>2</sub> HPO <sub>4</sub> . . . . .	0 50 gm.
KH <sub>2</sub> PO <sub>4</sub> . . . . .	0 50 "
MgSO <sub>4</sub> . . . . .	0 20 "
NaCl . . . . .	0 01 "
FeSO <sub>4</sub> . . . . .	0 01 "
MnSO <sub>4</sub> . . . . .	0 01 "
Peptone . . . . .	5 00 "
Carbohydrate . . . . .	30 00 "
H <sub>2</sub> O . . . . .	1,000 00 cc.

A solution containing a double concentration of mineral salts and peptone was made up in the experimental flask and sterilized for 45 minutes at 10 pounds steam pressure. In another flask an equal volume of double concentration sugar solution in water was sterilized. The two solutions were mixed in the experimental flask just previous to inoculation. The medium prepared in this manner has an acidity corresponding to pH 6.7. The various flasks were each inoculated with 10 cc. of a 24 hour culture of the organism growing in maize mash. The flasks were then incubated at 36°C.

*Estimation of Volatile Acids.*—It has been shown by independent workers (Reilly and others (2), Speakman (3)) that the volatile acids produced during the normal fermentation are acetic and *n*-butyric. Reilly also indicated the presence of a third acid which is only very slightly volatile in steam. Knowing, therefore, the number and nature of the constituent volatile acids it was possible to analyze quantitatively the mixture of acids by means of the Duclaux method (4). The criticisms of this method made by Upson and others (5) apply only in part to work in which the number and nature of the acids present are known. Their criticism

of the fundamental principle of the method, namely that each acid in a mixture comes over according to its own distillation constant, has not been supported by other investigators; *e.g.*, Gillespie and Walters (6).

The simple form of distillation apparatus originally described by Duclaux was used to obtain the table of constants given in Table I. The same apparatus was used throughout the experimental work. For purposes of comparison the figures contained

TABLE I.  
*Distilling Constants.*

Fraction	Formic acid		Acetic acid		Butyric acid	
	Duclaux	Present experiment	Duclaux	Present experiment	Duclaux	Present experiment.
<i>cc</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
10	5 9	5 9	7 4	8 3	17 6	16 6
20	12 2	12 5	15 2	16 6	33 6	31 4
30	19 0	19 6	23 4	25 1	47 5	45 1
40	26 4	27 5	32 0	33 8	60 0	57 5
50	34 4	35 9	40 9	43 1	70 6	68 3
60	43 2	45 0	50 5	52 8	79 5	77 7
70	52 8	55 4	60 6	63 0	86 5	85 5
80	64 6	67 4	71 9	74 0	92 5	91 5
90	79 6	81 7	84 4	86 1	97 0	96 8
100	100 0	100 0	100 0	100 0	100 0	100 0
Percentage of original in distillate.	59 0	64 5	80 0	80 3	97 5	97 5

in Table II of the work of Duclaux are included in Table I. Before proceeding further in the research it was shown by working with known mixtures and the above constants, that it is certainly possible to analyze a mixture of volatile organic acids with the accuracy usually ascribed to the method. In all the experiments the composition of the volatile acid fraction was determined by the algebraic method.

#### EXPERIMENTAL.

Before proceeding to a study of the fermentation of some of the rarer sugars it was necessary to obtain more complete infor-



mation regarding the production of acids during a normal fermentation. Mention has already been made of the presence of some unknown non-volatile acid or acids. An attempt was therefore made to identify this fraction, and to correlate the rate of its formation with the production of gas and neutral products of the fermentation. Reilly and coworkers (2) have reported on this point, but their data, illustrated by curves, require some correction. The total acidity curve of the medium during the third phase of the fermentation, *i.e.* from about the 20th hour to the end, is not a straight line.<sup>1</sup> There is a gradual rise in acidity which may continue for several hours after gas production has ceased. This is a point of considerable theoretical importance.

TABLE II  
*Starch.*

Time after inoculation	Total acidity *	Duclaux distilling constants.										Acidity due to		
		10 cc	20 cc.	30 cc.	40 cc	50 cc	60 cc	70 cc	80 cc	90 cc	100 cc	Acetic acid *	Butyric acid *	Non-volatile acid *
hrs.	cc	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	cc	cc	cc
18	3 3	13 0	25 5	37 3	48 5	58 4	67 8	76 7	84 8	92 4	100 1	25 1	96 0	0 8
20	3 3													
23	2 65													
27	2 0	12.0	24 0	35 3	45 5	55 4	64 7	73 6	82 0	90 7	100 0	89 0	82 0	29 0
43	1 9	11 0	21.7	32 0	42 0	51 5	61 0	70 6	80 0	90 0	100 0	95 0	49 0	46 0
66	2 2	10.3	20 6	30 9	40 9	50.5	60 1	69.8	79 4	89 0	100 1	08 0	46 0	66 0

\*In this, as in subsequent tables, the acidity of the medium is expressed in terms of 0.1 N NaOH per 10 cc. of sample.

*Experiment I.*—A fermentation flask containing 1,500 cc. of 3 per cent maize mash was sterilized for 1½ hours at 10 pounds steam pressure. The medium was allowed to cool to room temperature, and was then inoculated with 20 cc. of an active culture. At intervals during the fermentation the acidity of the mash was determined by titration, and 110 cc. portions were analyzed by the Duclaux method. The fermentation was quite normal, and the results are summarized in Table II, and represented by curves in Fig. 1.

The results from this experiment show that during the first phase of the fermentation the rapid rise in acidity is due almost

<sup>1</sup>*Cf.* Reilly and coworkers (2), p. 231; also Speakman (3), p. 322.

entirely to the production of acetic and *n*-butyric acids. The accumulation of non-volatile acid is more marked during the second phase, and it continues until after the production of gas has ceased. The increase in the acidity of the medium under these conditions was more clearly shown by analyses of sluggish fermentations. In these the amount of residual carbohydrate is often considerable, and the rise in acidity is proportionately greater than that which occurs after the close of a rapid and normal fermentation. By correlating these results with those contained in a previous report (3) we find that during the second phase, when the non-volatile acid is accumulating, the volatile acids, particularly butyric, are being reduced and oxidized with the rapid formation of acetone and butyl alcohol.

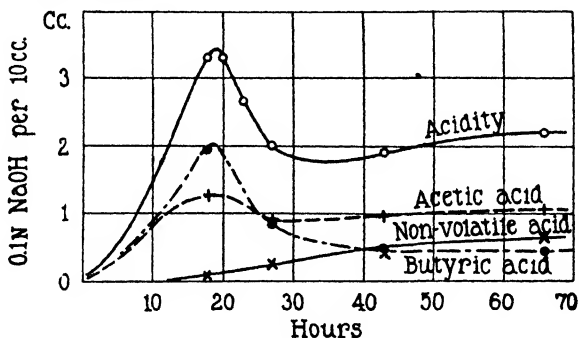


FIG. 1. Fermentation of maize mash.

During a maize fermentation the starch is hydrolyzed to glucose by exocellular enzyme action, and is utilized as such by the organism. A fermentation of this type is not strictly comparable with one in which a definite initial concentration of glucose or other sugar is used. In the maize fermentation the concentration of glucose rises during the first phase of the fermentation to a maximum, corresponding in time with the maximum acidity of the medium, and afterwards falls at about the same rate to zero. During the fermentation of a glucose medium the concentration of sugar gradually falls from the commencement of the fermentation.<sup>2</sup> In order, therefore, to obtain data relating to a normal

<sup>2</sup>Robinson (1), pp. 132, 147.

fermentation which would be strictly comparable with those of the rarer sugars the following experiment was performed.

*Experiment II.*—A flask containing 800 cc. of mineral salt solution plus peptone and glucose was sterilized and inoculated. The acidity of the medium was determined and analyzed at regular intervals. At the peak of gas production the medium was very slimy and difficult to distil. To overcome this difficulty the 110 cc. sample was neutralized with 0.1 N Ba(OH)<sub>2</sub>. The sample was then concentrated on the water bath to about 30 cc. and allowed to cool to room temperature. The acids were then reliberated with a slight excess of H<sub>2</sub>SO<sub>4</sub>, and the mixture was filtered. After restoring the volume of the filtrate to 110 cc the Duclaux analysis was made without frothing. The results from this experiment are condensed in Table III, and the curves in Fig. 2 are based on these data.

TABLE III.

*Glucose*

Time after inoculation	Total acidity	Duclaux distilling constants										Acidity due to		
		10 cc	20 cc	30 cc	40 cc	50 cc	60 cc	70 cc	80 cc	90 cc	100 cc	Acetic acid	Butyric acid	Non-volatile acid.
	cc	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	cc	cc	cc
hrs														
5	0 6													
22	3 0													
26	3 3	12 9	24 9	36 2	46 6	56 1	65 7	74 2	82 7	91 1	100 1	17 1	26 0	87
46	3 3													
51	2 9	10 8	21 0	31 3	40 9	50 3	60 0	69 3	78 9	88 8	100 1	30 0	50 1	10
70	2 4													
94	2 2	9 5	19 0	28 5	38 0	47 4	56 9	66 4	76 7	88 0	100 1	05 0	21 0	94
118	2 4													

The differences between the maize mash and glucose fermentations are reflected in the curves of Figs. 1 and 2. The fermentation of glucose is much slower, but the accumulation of non-volatile acid is most rapid in the first phase of the fermentation, whereas in the maize fermentation this is not the case. The maximum concentration of non-volatile acid in the glucose fermentation coincides with the maximum acidity of the medium in time, and after this period there is no marked change in the non-volatile acid present. After gas evolution has ceased there is a slow rise in total acidity, due entirely to production of non-volatile acid.

These results illustrate very definitely the changes in the biochemistry of the normal fermentation brought about by the initial high concentration of glucose and the substitution of peptone for maize proteins.

*Experiment III.*—An attempt was then made to isolate and identify the non-volatile acid products of the fermentation. 5 liters of a 5 per cent maize mash were inoculated and allowed to ferment to completion. The bulk was then filtered through cotton-wool, and a clear yellowish green solution, free from starch and insoluble protein, was obtained. This was evaporated down under reduced pressure, 15 to 20 mm., at a temperature

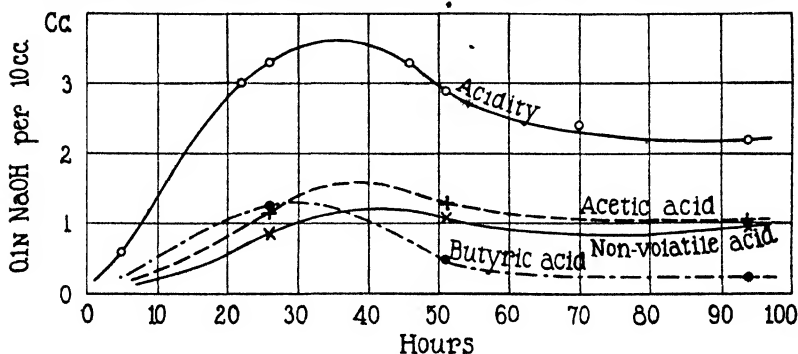


FIG 2 Fermentation of glucose.

of 40°C. A liter of distilled water was added, and the solution was again evaporated down to a thick syrup. During the last hour of the evaporation the temperature was raised to 60°C. For the details of this method for the removal of volatile acids we are indebted to a recent paper by Stephenson and Whetham (7). The final syrup was light brown in color and very viscous. It was then diluted down to 200 cc with distilled water, and extracted with ether in a continuous extractor for a period of 24 hours. The ether layer was separated, and added to 200 cc of distilled water. After removing the ether by distillation the aqueous solution of non-volatile acid was boiled under a reflux condenser for 2 hours with an excess of  $\text{ZnCO}_3$ . The solution was then reduced to a smaller volume on the water bath and allowed to stand in a shallow dish at room temperature.

The crystals obtained had the same structural characteristics as a sample of pure zinc lactate which was freshly prepared. We obtained a positive result when small amounts of the material were submitted to the Uffmann and the Hopkins-Cole tests. By oxidation with acidified potassium permanganate solution a sample of the material was converted into acetaldehyde. The

production of traces of lactic acid by this bacillus is in accordance with the work of Winogradsky (8) and Störmer (9) who have investigated the biochemistry of other members of the *amylobacter* group. We have satisfied ourselves that the lactic acid is formed from the sugar in the medium, and not from the protein, by studying the products of the fermentation of a medium in which urea was the only source of nitrogen.

### *The Fermentation of Rarer Sugars.*

*Experiment IV.*—This experiment was performed to discover whether in fermentations which we have termed abnormal the characteristic neutral and acid products are formed. The sugars investigated were xylose, arabinose, galactose, dulcitol, and mannitol. Erlenmeyer flasks containing

TABLE IV

		Test applied.	Xylose	Arabinose	Galactose	Mannitol
Neutral products.	Acetone	Rothera's.	++	+++	+	+
	Butyl alcohol	Solubility	+	++	+	+
Acid products; qualitative.	Formic.	AgNO <sub>3</sub>	—	—	—	—
		HgCl	—	—	—	—
	Acetic.	Duclaux	+	+	+	+
	Butyric.	"	+	+	+	+
	Lactic.	Hopkins-Cole	+	+	+	+
		Uffelmann	+	+	+	+
Acid products, quantitative	Acetic		cc	cc	cc	cc
	Butyric		1 6	1 9	2 2	1 47
			1 1	0 7	2 86	1 85
	Lactic		1 1	1 2	1 04	0 98
Total acidity			3 8	3 8	6 1	4.3

150 cc of media were sterilized and inoculated. Each pair of flasks contained one of the above sugars in 3 per cent concentration. Daily observations were made on the external appearance of the flasks, and they were allowed to remain in the incubator until all fermentations had ceased. The titratable acidity of each flask was then determined. From each a 110 cc sample was withdrawn and neutralized with 0.1 N Ba(OH)<sub>2</sub>. These solutions were separately distilled to obtain the neutral products which were collected in about 25 cc. of distillate. Each distillate was tested for acetone and butyl alcohol. The residues were concentrated on the water bath and cooled. To each a sufficient volume of dilute H<sub>2</sub>SO<sub>4</sub> was added to reliberate the acid products. After restoring the volume to 110 cc. with

water each was submitted to a Duclaux analysis. The volatile fractions were tested for formic, acetic, and butyric acids. The residues were extracted with ether, and qualitative tests made for lactic acid. It is not necessary to describe these various tests in detail, but the qualitative and quantitative results from the experiment as a whole are given in Table IV.

The results from the experiment contain several interesting facts. Both pentose sugars are fermented and give rise to normal products. There was no evidence whatever of formic acid in the medium. Arabinose is fermented more rapidly and more completely than xylose. During the first 40 hours of the galactose fermentation there was a brisk evolution of gas, but very rapidly this was reduced, and the fermentation became sluggish and prolonged. The final titration figure is very high, and the medium is particularly rich in volatile acids. Of the two alcohols, mannitol and dulcitol, only the former is fermented.

The fermentation products differ markedly in the amounts of volatile acid produced and in the ratio between acetic and butyric acid present. Attention is called to the fact that in the pentose fermentations the acetic acid figure is much higher than the butyric acid, whereas in the other fermentations the reverse is the case. In these fermentations, as also in the glucose fermentation, the amount of non-volatile acid produced is fairly constant, representing 1.0 to 1.2 cc. of 0.1 N acid per 10 cc. of medium. The writer is of the opinion that this concentration of lactic acid is very near to that which is toxic to the bacillus.

*Experiment V.*—Large flasks of media containing the various sugars or alcohols were sterilized and inoculated. At regular intervals large samples were taken for analysis by the Duclaux method. Each type of fermentation was repeated three times in order to eliminate, as far as possible, the chance of obtaining results which are not characteristic. The results given in Tables V to VIII are, therefore, typical cases from a long series of fermentations. The curves in Figs. 3 to 6 are based on the same data.

The results for this group of experiments enable us to form conceptions regarding the fermentations which it would be almost impossible to formulate from the data relating only to final products. We find that those fermentations which appeared to be similar, judging by the data of the last experiment and work previously reported (1), have several important distinguishing features. The pentose fermentations differ from the normal

glucose fermentation in the relationship existing between the acetic and butyric acid curves, the production of acetic acid being much greater. They differ in themselves in several respects. The fermentation of arabinose is more rapid and more complete than

TABLE V  
*Arabinose.*

Time after inoculation	Total acidity	Duclaux distilling constants										Acidity due to		
		10 cc	20 cc	30 cc	40 cc	50 cc	60 cc	70 cc	80 cc	90 cc	100 cc	Acetic acid	Butyric acid	Non-volatile acid.
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	cc	cc	cc
hrs	cc													
38	6 4	11 0	22 0	32 3	42 1	51 8	61 2	70 4	79 6	89 2	100	3.1	1 6	1 7
67	5 8													
86	5 5	10 6	21 2	31 2	41 0	50 4	60 0	69 4	79 1	88 8	100	2 7	1 1	1 7

TABLE VI  
*Xylose*

Time after inoculation	Total acidity	Duclaux distilling constants										Acidity due to		
		10 cc	20 cc	30 cc	40 cc	50 cc	60 cc	70 cc	80 cc	90 cc	100 cc	Acetic acid	Butyric acid	Non-volatile acid.
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	cc	cc	cc
hrs	cc													
18 0	2 0													
24 0	2 9													
42 0	4 3	13 1	25 4	36 8	47 4	57 3	66 6	75 7	83 9	92 0	100	1 5	1 95	0 85
49 0	4 5													
66 0	4 6													
71 0	4 7	12 5	24 1	35 2	45 5	55 4	64 8	74 1	82 8	91 1	100	1 95	1 85	0 90
90 0	4 7	12 1	23 7	34 5	44 8	54 9	64 3	73 6	80 1	90 9	100	2 15	1 85	0 70

that of xylose. Associated with this general distinction we find evidence of a more vigorous utilization of the volatile acids produced. This observation has greater interest when we remember that in the chemical sense xylose is associated with glucose, and arabinose with galactose. The results from the biological study of these sugars, in as far as they relate to rapidity and complete-

ness of utilization, have reversed this grouping. This matter will be discussed in greater detail at the end of the paper.

TABLE VII.

*Galactose.*

Time after inoculation	Total acidity	Duclaux distilling constants										Acidity due to:		
		10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.	Acetic acid	Butyric acid	Non-volatile acid.
hrs.	cc.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	cc.	cc.	cc.
20 0	2 5													
22 5	3 0	11 9	23 2	33 8	44 1	54 0	63 2	72 3	81 0	90 0	100 1	31 0	96 0	72 0
25 5	3 3													
42 0	5 3	12 6	24 5	35 8	46 4	56 3	65 7	74 5	83 0	91 4	100 2	00 2	15 1	15 1
44 0	5 2													
48 75	5 3													
67 0	5 65	12 7	25 0	36 4	47 1	57 1	66 7	75 4	83 8	91 9	100 2	30 2	90 0	40 0
92 0	6 4	12 9	25 0	36 7	47 2	57 2	66 7	75 4	83 8	91 7	100 2	30 2	90 0	16 1

TABLE VIII

*Mannitol*

Time after inoculation	Total acidity	Duclaux distilling constants										Acidity due to:		
		10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.	Acetic acid	Butyric acid	Non-volatile acid.
hrs	cc	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	cc	cc	cc.
43 5	3 65	14 6	28 0	40 6	51 6	61 7	70 8	79 2	86 7	93 5	100 0	80 2	20 0	64 0
48 5	3 90													
67 5	4 40	14 4	27 9	40 0	51 5	61 6	70 9	79 2	86 7	93 3	100 0	96 2	70 0	73 0
75 0	4 45													
91 5	4 75	14 4	27 5	39 7	50 9	61 1	70 3	78 7	86 2	93 2	100 1	13 2	80 0	83 0
97 5	4 90													
116 0	5 00	13 8	26 6	38 8	49 9	60 1	69 4	77 9	85 8	93 1	100 1	45 2	99 0	56 0
142 0	5 30													
166 0	5 35	14 0	25 9	39 0	50 1	60 3	69 6	78 3	88 4	93 2	100 1	46 3	10 0	78 0

During the first 40 hours the fermentation of galactose is apparently normal. The rate of gas production and the rise in



acidity are very similar to those in the glucose fermentation, although on the whole more vigorous. For the next 24 hours the total acidity shows little change, and at the commencement of

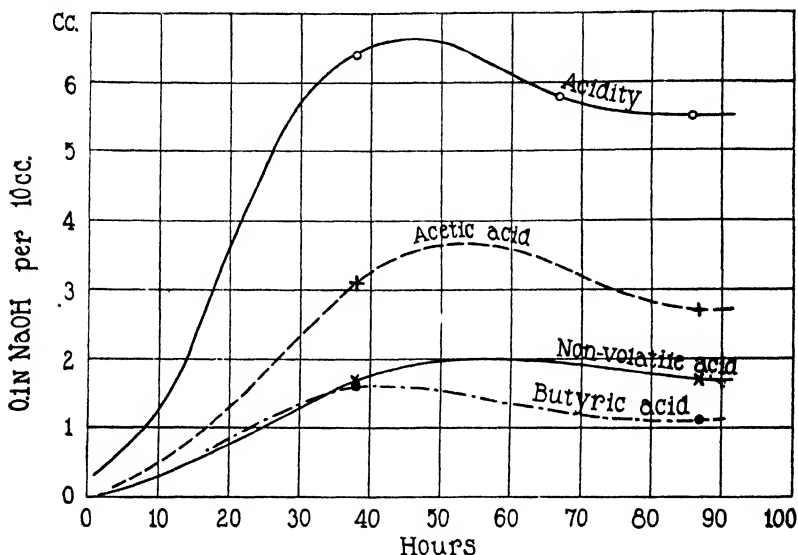


FIG. 3. Fermentation of arabinose.

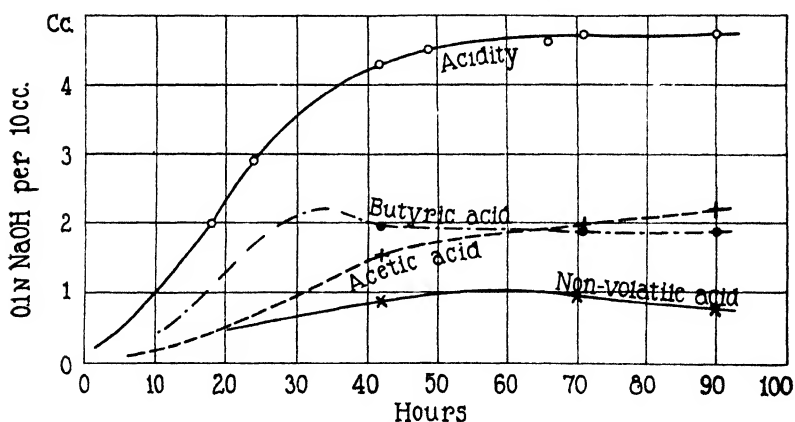


FIG. 4. Fermentation of xylose.

this period the fermentation very rapidly changes to a slow and indifferent one. The accumulation of butyric acid and acetic acid continues, showing the absence of any vigorous utilization

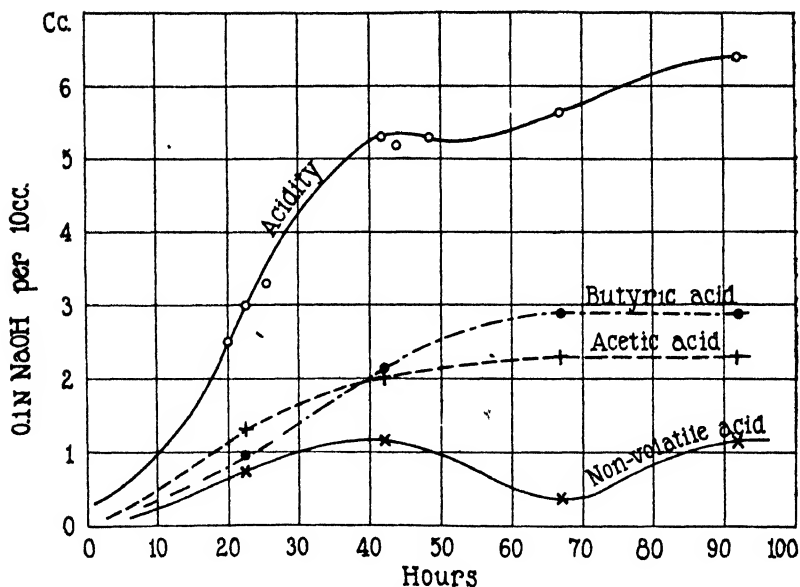


FIG. 5. Fermentation of galactose.

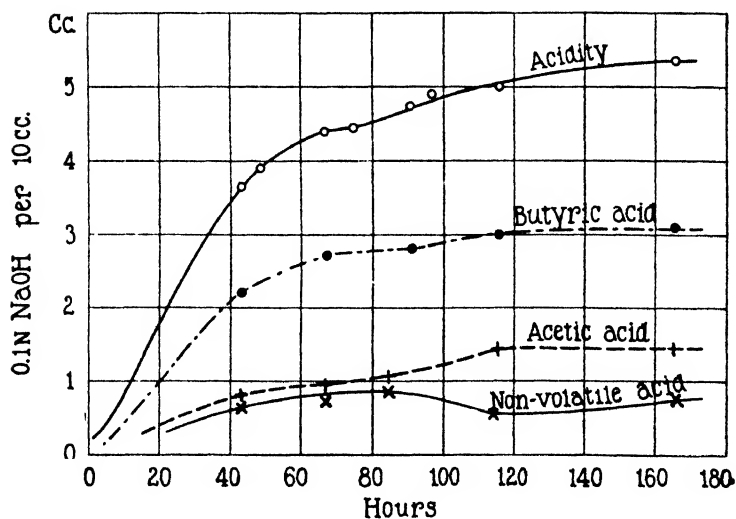


FIG. 6. Fermentation of mannitol.

of these acids. Of still greater interest is the lactic acid curve. For about 24 hours the concentration of lactic acid falls; *i.e.*, until about the 68th hour of the fermentation. At this point three changes occur. The evolution of gas and the production of volatile acid cease, and almost at the same time the lactic acid curve begins to rise once more, and this continues for another 24 hours. We can only regard this last period as a true lactic acid fermentation, and the galactose fermentation as a whole is characterized by the vigorous production and utilization of lactic acid rather than by the formation of acetone and butyl alcohol.

The fermentation of mannitol is also distinct in several respects. Again we observe the vigorous production of acids and gas during the first 30 hours, and once more this period is followed by a slow fermentation. The most characteristic feature of the mannitol curves is the large amount of butyric acid produced. Apparently this intermediate is untouched, whereas the curves for acetic acid and lactic acid show that production and utilization are both taking place. Remembering the chemical relationship between mannose and mannitol we are justified in concluding that the changes in the latter fermentation from the normal are due to the change from  $-\text{CHO}$  to  $-\text{CH}_2\text{OH}$ . The detail of the biological change is at present a matter for discussion rather than of fact, and for this reason will not be considered further at this point. Mention has already been made of the fact that dulcitol is not fermented by this organism. We cannot attribute this fact to the terminal groups of the molecule, but an explanation may be forthcoming if we compare groupings 2, 3, 4, and 5 in the mannitol and dulcitol molecules.

#### *Biochemistry of the Normal Fermentation.*

Before discussing the influence of the structure of the utilized carbohydrates on the products of the fermentation, it is necessary to state briefly our views regarding the biochemistry of the normal glucose fermentation in the light of new experimental data. The diagram of reactions contained in Fig 7 is an attempt to correlate and interpret available information. In view of the complexity of the fermentation such an attempt can only be regarded as provisional.

It is unnecessary to repeat in this discussion the evidence for the existence in the fermentation products of the compounds

mentioned in Fig. 7. The suggested oxidation of lactic acid and acetic acid is in harmony with the biochemical mechanism of other fermentations, and is supported by experimental evidence contained in this paper. Such oxidation affords an explanation of the origin of a considerable portion of the gas produced. The most important point in the scheme is the suggested origin of the organic acids. Whereas Neuberg and Arinstein (10) and others have suggested that they are derived from some common intermediate, acetaldehyde or pyruvic acid, the present writer considers that they are produced by the cleavages and oxidation of the sugar molecule. From the hexoses the initial products are 4C plus 2C and 3C plus 3C compounds, and from the pentoses 3C plus 2C and 4C plus 1C compounds. The quantitative figures

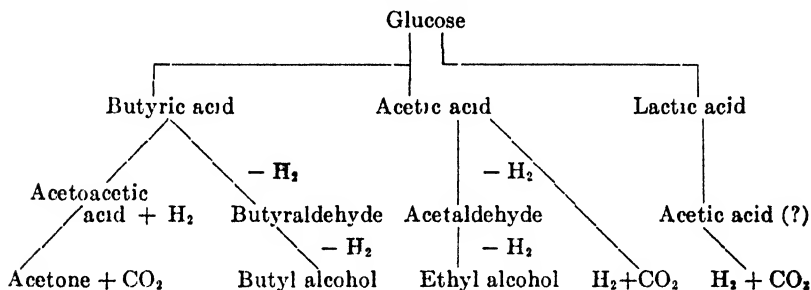


FIG. 7.

in this paper which relate to the glucose, galactose, and mannitol fermentations afford strong evidence that in these cases the reactions involving the production of butyric and acetic acids are intimately associated. On the other hand, in these fermentations the production of lactic acid often proceeds alone, particularly towards the close of the fermentation. The pentose fermentations show a close connection between the production of lactic acid and acetic acid. There is no independent production of lactic acid at the close of these fermentations, but changes occur in the concentrations of both lactic and acetic acid after the butyric acid reactions have stopped.

*The Relationship between Structural Formulas of Carbohydrates and the Biochemistry of the Fermentation.*

This problem in its general aspects is one of great importance and interest to the microbiologist. In as far as bacteria in general are concerned it has been studied largely from one point of view

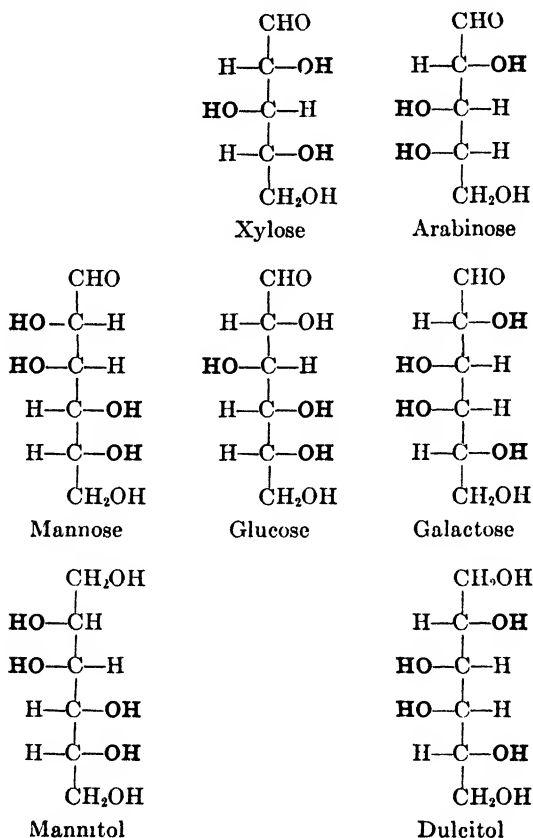


FIG. 8.

only; namely, does a particular type utilize certain carbohydrates with the production of its characteristic end-products? A great need exists at the present time for more detailed and exhaustive studies of the biochemistry of individual species.

In Fig. 8 are arranged the formulas of the carbohydrates which have been studied in this investigation. Accepting for the mo-

ment the conclusions regarding the biochemistry of a normal glucose fermentation, are we able to explain the characteristics of the abnormal fermentations in terms of molecular configuration in the carbohydrates utilized?

The fermentation products are the same in all cases; therefore our problem is one in which we have only to consider the structure of the various sugars and the rates of formation and consumption of these products. If we first of all limit our observations to the fermentation of the three aldohexoses we shall eliminate the possible distinctions which are due to differences in the number or nature of groups in the sugar molecule. Within this small group of fermentations any distinctions must be due to differences in the relationship between the  $-OH$  groups in the molecules of mannose, glucose, and galactose. The generally accepted view regarding the formation of lactic acid from glucose is that the process is essentially one of intramolecular rearrangement in which molecules of water are involved. At some stage in this process the break in the sugar molecule occurs. Assuming that some similar processes are responsible for the production of the organic acids by this organism, the experimental evidence indicates that, in order that cleavage may occur at some particular place in the molecule, it is not essential that the neighboring  $-OH$  groups shall be adjacent. Is it not probable, however, that the changes leading to breakages in the chain proceed with greater ease and rapidity in those cases in which suitably placed adjacent  $-OH$  groups are to be found in the sugar molecule? This being the case, it follows that the total rate of the fermentation, and in this group of fermentations where alternative cleavages are possible, the relative rates of different reactions are influenced by the number and position of such groups. We therefore expect, from the structure of the three hexoses which we are considering, that mannose will be fermented more rapidly than glucose or galactose, owing to the fact that mannose contains two pairs of adjacent hydroxyls. Experimental evidence has shown this to be the case.<sup>3</sup> Furthermore, we should expect: (a) glucose and galactose to be fermented with equal rapidity, (b) the glucose fermentation to be essentially one in which 4C and 2C compounds are produced, and (c) the galactose fermentation to be one in which the production and utilization of lactic acid are prominent. It is impossible to make justifiable comparisons between the rapidity and completeness of the two fermentations, owing to the fact

that the galactose fermentation is largely composed of the production and utilization of lactic acid, the acid which is most toxic to the organism. The curves show, however, that during the first 40 hours of the two fermentations, *i.e.* before the first high concentration of lactic acid is reached in the galactose one, the two sugars are utilized at approximately the same rate (1). Later, the galactose fermentation becomes slow, and there is a large amount of residual sugar. We find that our conclusions, derived from the structure of the hexoses and a knowledge of the glucose fermentation, are in general harmony with the experimental results.

We shall now consider very briefly the structure of the pentose sugars, and endeavor to show a connection between certain features of the molecules and the characteristics of the fermentations. In the arabinose molecule, but not in xylose, there is one pair of adjacent  $-OH$  groups. We expect therefore, acid production to be the more rapid in the arabinose fermentation. In both fermentations, but more especially in that of arabinose owing to the particular position of the adjacent  $-OH$  groups, we should expect a vigorous production and utilization of lactic and acetic acids. Furthermore, owing to this particular form of acid production, the fermentations, similar to the galactose fermentation with regard to lactic acid, probably become sluggish after a few hours of vigorous fermentation, and the sugars are only partially utilized. All these statements are in accord with experimental results from the pentose fermentations. We have also shown that butyric acid is produced, but no formic acid, indicating an endocellular decomposition of the latter acid into  $H_2$  and  $CO_2$ . The reaction responsible for the production of these two acids must involve the terminal  $-OH$  group.

The results from fermentations of the alcohols corresponding to the common hexoses are of great interest. It has been pointed out that, "Inasmuch as enol formation is not possible in the alcohol series corresponding to the hexose series, a closer relation should exist between the space relations of the alcohol and the cytoplasm of the bacterium to insure fermentability than in the hexose series where a common enol furnishes a plausible reason for the natural utilizability of the tautomerids" (Kendall and others (11)).

Applying the theory regarding the influence of adjacent hydroxyls to the two alcohols studied we should expect: (a) mannitol

to be fermented more rapidly than dulcitol, (b) the mannitol fermentation to be characterized by a vigorous production of butyric acid and acetic acid, and (c) the dulcitol fermentation to be essentially a lactic acid one. In as far as the mannitol fermentation is concerned our expectations are in accord with the experimental data, but the bacillus is unable to ferment dulcitol. Therefore, although the theory regarding the influence of adjacent hydroxyls adequately explains the great majority of these experimental results, in the case of dulcitol it is of no apparent assistance. Further investigation may enable us to show that this conflict is apparent rather than actual, and due to other factors. On the other hand, a theory which correlates and explains the great majority of our present experimental data may have to be modified or even abandoned in the light of new knowledge.

## SUMMARY.

1. The non-volatile acid produced from starch and sugars by *Bacillus granulobacter pectinovorum* has been identified as lactic acid.

2. The formation of acetic, butyric, and lactic acids has been quantitatively studied during the fermentation of starch, arabinose, xylose, glucose, galactose, and mannitol.

3. The biochemistry of the production of the acid and neutral products of the fermentation has been discussed.

4. A theory has been advanced to account for the influence of the structure of the fermentable sugars on the fermentation products.

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## RHYTHMIC BANDING OF PRECIPITATES (LIESEGANG'S RINGS).

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Rhythmic banding is a common phenomenon in biology. Examples are: the lamellar rings around the Haversian canals in bone; the pigmentation of the iris; the color of butterflies' wings; the growth rings of trees; the agate structure of crystals; the shells of mollusca; the bands of starch grains and of many seeds.

Rhythmic banding of precipitates was first discussed by Raphael E. Liesegang (1), hence the name "Liesegang's rings." He observed that when a solution of silver nitrate is placed on a gelatin gel containing dilute potassium bichromate, rhythmic banding of the precipitate occurs. If the solutions are in a dish or on a plane surface the precipitate is in concentric rings, if in a test-tube, superimposed layers of the precipitate are formed. Numerous explanations have been advanced, but none is generally acceptable. Ostwald's explanation (2), which postulated a metastable condition, was accepted until Liesegang, Bechold (3), and Hatschek (4) proved it untenable.

Bechold (3) advanced the theory that the band precipitate is soluble in the products of the reaction, and new bands are formed only when the concentration of the reaction products is so dilute that it will not interfere. It has been shown, however, that the effect of the by-products is negligible (5).

Bradford (6, 7) in 1916 put forward a theory which would explain ring formation by adsorption. Since then he has published much work to support his views. Holmes (8) has also advanced a theory similar to Bradford's, but attributes the formation to ionic activities, instead of adsorption effects of the colloidal particles of the precipitate. Bancroft (9), quoting Hatschek, states that all theories fail to account for the specific effect of the gel. Bradford,<sup>1</sup> however, accounts for this by saying that the value of  $N$  in von Weimarn's formula is influenced by the gel. We do not think this explanation is adequate. The explanation given by Bradford and by

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<sup>1</sup> Bradford (7), p. 17.

Holmes, as far as the process of diffusion is concerned, however, seems adequate.

Fischer and McLaughlin (10), on theoretical grounds, think that ring formation is possible only under conditions where a semipermeable membrane is formed by the precipitate. If the precipitate rapidly crystallizes, it is permeable and therefore the precipitation is continuous. If it be temporarily impermeable, rings are formed. If the precipitate first formed is amorphous and "solvatisierte," a ring is formed. Then in time the precipitate becomes "desolvatisierte," solid and permeable, and the penetrating ion passes through. Ring formation is repeated. There is little evidence that the precipitate *per se* is impermeable, although the precipitate together with the gel may in many cases form an impenetrable layer. This seems to account in some cases for the difference in ring formation in agar and gelatine gels. Where such an impenetrable membrane is formed, however, it remains so, and prevents further ring formation.

Recently, the senior author (11) advanced an explanation of the rhythmic banding of precipitates, which accounts for the effect of the gel. The main points of this explanation are: banding in precipitation is the normal and universal course, but in water solutions, due to secondary causes such as changes in specific gravity, violent combination, concentration, etc., which stir up or obscure the reaction medium, the bands are destroyed as rapidly as they are formed. The functions of gel in banding are: (1) to fix the precipitate where it is formed and thus to keep the rings visible, and (2) to modify the rate of diffusion of the reacting ions so that favorable conditions for banding are provided. This occurs through the influence of the colloid in damping the vibration of the reaction, and in lessening and limiting the rate of diffusion of one ion more than another.

The process of this interrupted growth theory of ring formation, using bichromate gelatine and silver nitrate as a type, occurs as follows: the bichromate in the gelatine is relatively fixed and diffuses slowly. When a solution of silver nitrate is added, silver bichromate (whether chromate is formed is immaterial in this case) is formed and a clear zone, practically free from chromate, results in the gelatine, due to the attraction of the chromate to the silver. This we have called the zone of influence of the silver ion. Beyond the zone the chromate is fixed (relatively) and remains so unless an attraction force is exerted. The silver nitrate now diffuses through the precipitate and through the clear zone until it approximates the chromate in the gelatine sufficiently close to

exert an attraction force which again draws the chromate and forms another ring and clear zone. At the same time the chromate ion attracts the silver ion and the ring is formed where the forces are balanced. When this precipitate is formed there is a zone relatively free from chromate on one side of the precipitate, and a corresponding zone of lessened silver concentration on the other. This leaves a period (season) of no growth, and the new period of growth (ring formation) commences when the silver, which diffuses more rapidly than the chromate under these conditions, passes through the precipitate and the zone of influence and comes again in contact with the chromate ion.

There is some evidence that all chemical action may be periodic; *e.g.*, Ostwald (12) found that if the rate of evolution of hydrogen gas, when HCl acts on one of the allotropic forms of chromium, during the action be plotted as ordinate against time as abscissa, the curve will show alternating periods of rapid and slow evolution. The curve in a measure corresponds to ring precipitation.

The explanation we present does not differ materially from that of Holmes, except that it accounts specifically for the action of the gel. Bradford's explanation is also very similar, but we think he stresses unduly the importance of adsorption. While it is generally accepted that the adsorption of ions from solution by a precipitate in the process of its formation is the rule, there is no reason to attribute more importance to it in banded than in continuous precipitates. Also the factor of adsorption after precipitation we think is overestimated. While Bradford has done much excellent work to support his theory, this work could be used equally well to support any of the other theories. Surface energy always exists, sometimes negative, sometimes positive, and banding may occur in both conditions; adsorption, therefore, cannot be a prime factor in initiating or controlling the reaction. The influence of adsorption would also seem much less than that of the ion, since adsorption varies inversely as the fourth power of the distance, while chemical attraction varies inversely as the square of the distance (13).

Bradford uses the work of von Weimarn (14) to support his theory. In this work von Weimarn shows that the form of the precipitate, *i.e.* whether crystalline or colloidal, is dependent on the number of crystallization

centers. If in the double decomposition reaction,  $AB + CD = AC + BD$ , the nature of the precipitate  $AC$  will be governed by the formula

$$N = \frac{P}{L} \times K_{AB} \times K_{CD} \times K_{BD} \times K_{AD} \times Z$$

$N$  = the number of crystallization centers, which we may call the dispersion coefficient.

$P$  is the number of equivalents of the precipitate  $AC$  which must be deposited out of each liter in order that its concentration be reduced to

$L$ , the solubility of the precipitate.

$K_{AB}$ ,  $K_{CD}$ , etc., are coefficients expressing the complexity of the various components.

$Z$  is the viscosity of the reaction medium.

For substances having simple chemical structure, such as we are dealing with, and which are not associated within the solution, the above formula may be simplified without significant error to

$$N = \frac{P}{L} \times Z$$

and since von Weimarn found this law to hold in water solutions, the most important factor in ring formation in gels seems to be  $Z$ , or the viscosity of the gel. This is the factor which Bancroft states has not been sufficiently considered in other theories.

*Reasons for Stating that Banding Is the Normal Method of Precipitation.*—Experiments cited by Liesegang (15), which we have confirmed, seem to us to speak against the adsorption theory; *e.g.*, if 10 per cent gel, containing  $\text{Na}_3\text{PO}_4$  (0.5 per cent), be poured on a glass plate and allowed to dry, definite banding is obtained. Bechold makes similar claims and also states that freezing water produces rings (9). This we have also observed. In this case, unless adsorption be used in a very loose sense, the banding is due to crystallization. If it is claimed that crystallization is explained by adsorption, then it has no especial significance in rhythmic banding.

Rohonyi (16) has shown that gelatine layers, on freezing, form in rings. Accepting Ostwald's explanation, his results support it. When tubes with a thin layer of gelatine are frozen, layers are formed, clear and dark. The clear layers are ice, the dark, gelatine. These rings are quite similar to Liesegang's.

Again, bands may appear in air, water, or in gels. Holmes (8) quotes an experiment of band formation in a glass tube where air took the place of the gel. We are able to confirm this as follows:

Concentrated HCl was placed in a small flask, and this connected with an upright glass tube 75 cm. long and 2 cm. in diameter. The upper end of the tube was plugged with cotton and a few cubic centimeters of strong ammonia water were dropped on the cotton. On standing overnight practically the whole length of the tube was covered with bands of ammonium chloride.

By rhythmically forcing air into the flask containing HCl (concentrated) so as to change the rate of diffusion, bands are formed on the glass at different places than if the diffusion took place naturally; in fact, by such manipulation, one may place the bands in any desired location. This action resembles that given by Burton and Bell (17), in which rings were formed in gelatin under tension. The gel was stretched longitudinally, the rings were elliptical, the major axis in the direction of the stretching force. In our work, after several distinct single bands were formed, there were zones of numerous very fine bands instead of the single bands. If the  $\text{NH}_3$  is drawn down into this clear space, fine bands result.

Again, if solutions of  $\text{AgNO}_3$  and KI be placed in an open test-tube and allowed to stand until evaporation occurs, distinct bands may reach to the top of the tube. Also, if a cholesterol in chloroform be placed in a tube and evaporated, definite rings are formed. Slow evaporation of many other solutions will produce rings, which seems due to capillarity and surface tension. This, however, may be quite distinct from the rhythmic banding in precipitation, although capillarity in gels undoubtedly plays a rôle. Perhaps because evaporation rings are of more common occurrence than precipitation rings, they have received less attention.

Dreaper (18) has shown that when precipitation occurs in capillary tubes, preferably set in a horizontal position, and care being taken to avoid stirring, etc., stratified precipitation may be the usual result in water solutions. In this way he prepared bands of lead chloride, lead sulfate, silver salts, barium sulfate, and many others.

We have been able to repeat this with 0.1 N  $\text{AgNO}_3$  and 0.05 N  $\text{K}_2\text{Cr}_2\text{O}_7$ . If the silver solution be drawn into a capillary tube or small pipette, and introduced with care into the bichromate solu-

tion, one often finds definite banding. These bands are seen better with a magnifying glass, and are sometimes difficult to prepare. Dr. L. F. Shackell, who first called our attention to this, found that a small bubble of air between the solutions aided ring formation. Again, if the chromate solution be poured on a glass plate and allowed to dry, and a small crystal of  $\text{AgNO}_3$  added and moistened carefully, definite ring formation may be seen.

The banding of precipitates, therefore, seems to be a universal phenomenon, and under proper conditions may be visible in all cases. This may appear opposite to the statement of one of us in a previous article (11) where it was said that, if lead acetate be used instead of silver nitrate on a bichromate gelatine gel, no ring formation occurs. Bradford (19) is correct in stating that with proper concentrations, rings of lead chromate in gelatine may be prepared. Many incorrect statements have crept into the literature of rhythmic precipitation, and the reason seems to be that rings in some cases are more difficult to prepare in visible form. But since rings may be rendered visible in practically every precipitation, any statement regarding their presence or absence is relative only. To illustrate further such incorrect statements we may quote the following from Bancroft.<sup>2</sup> "The reactions between silver nitrate and potassium bichromate give rings or layers in gelatine but not in agar, while lead nitrate and potassium chromate give layers in agar but not in gelatine." Bradford (5) also in one of his latest articles says: "It has been found that neither silver chromate or bichromate form bands in agar gels" and in discussing the theory advanced by one of us, says: "It is not sufficient to explain why bands form in gelatine and not in agar." All such statements require qualification, and some of them have been corrected later by these authors. When we adopt the view that rhythmic precipitation is the normal process, the explanation is more easily understood.

Bands form readily in agar as the following experiments will show.

Mix equal parts of 1 per cent agar and 0.02 N potassium bichromate in a test-tube. When a gel has formed, pour 0.1 N silver nitrate on top. After 12 hours twenty-three bands were counted in a space of 2 cm. beginning 2.5 cm.

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<sup>2</sup> Bancroft (9), p. 259.

from the surface of the gel; these were very delicate and better seen by transmitted light. To show rhythmic banding, time and concentration are important functions as they regulate the rate of diffusion. It is for this reason that the concentration of the penetrating ion is greater. Also the test-tube method may show banding that is not visible on surfaces.

No new chemical process is involved in the formation of rings. The colloid is simply an interfering body, modifying the intensity of the reaction, and calming or damping the reaction field by immobilizing the precipitate where it is formed. In some cases where the gel and the precipitate form an impenetrable membrane, or a membrane that is penetrable with difficulty, no ring formation is visible. If the physical state of the reaction medium remains calm in water solutions, as in the case of the ammonium chloride in air, and in precipitation in water solutions in capillary tubes, ring formation is seen to be the usual process. On plane surfaces precipitation rings are formed in circles somewhat similar to the rings formed by dropping a stone into a pool of calm water. If the pool be turbulent the rings are destroyed or twisted out of their course. Similarly, in an ordinary chemical reaction the local storm, caused by the reacting ions and by the difference in the specific gravity of the product formed and the solvent, disturbs the medium to such an extent that the rings are destroyed.

The opposite effect of a gel can be obtained by adding ether, alcohol, or acetone to the reacting medium. This causes in water solutions a marked increase in the turbulence of the reaction, apparently due to lowering of surface tension. There are cases, however, where the tendency to band is increased by the addition of these bodies to the gel; i.e., where the viscosity is so great that an impermeable membrane is formed. The addition of alcohol, by lessening the viscosity, changes permeability so that banding may occur. Sugar also is known to increase banding in such cases.

Liesegang (15) has recently pointed out a similar phenomenon in the case of silver chloride bands in gelatine. If to a gel containing silver nitrate, sodium chloride solution be added, no banding is visible. The reason seems to be that the penetrating Cl ion moves too rapidly in relation to the Ag ion, which may be partly combined with the gel. The precipitate, therefore, is continuous. If, however, silver bichromate bands are first formed, and then sodium



chloride be added, the chromate bands are replaced by chloride bands. The silver chromate here acts as a "form catalyser." The reaction involved is that used in the determination of chlorides by Mohr's method.

Besides the rate of penetration, however, there is another factor of importance, and this is the degree of mobility of the incarcerated ion; *i.e.*, the ion of the gel. The following experiment will illustrate.

Two tubes were made as follows:

Tube 1. Agar, 0.5 per cent.  
Pb acetate, 0.1 N.

Tube 2. Gelatine, 5 per cent.  
Pb acetate, 0.1 N.

When these had coagulated 10 per cent KI was poured on top. After 36 hours the iodide solution had penetrated about the same distance in each, but in the agar there were beautiful rings, while in the gel there was only a suggestion of ring formation.

The difference seems to be due to the nature of combination of the lead acetate in the two cases. In the gelatine it is more intimately combined and less moveable; in the agar it is practically free and moveable, and gets entangled in the agar net only when particles of the precipitate are formed. When, however, precipitation has descended several centimeters in the tube, rings become visible also in the gelatine. This is attributed to a change in the rate of the penetrating ion (I) which is now slower than where the precipitate is continuous. That rate of penetration, or rate of meeting of the ions, is important is readily shown by the methods of preparation of rings. To obtain the best results, the penetrating ion is always in greater concentration than the ion in the gel, *e.g.* if 0.05 N  $\text{AgNO}_3$  be placed in water solution on top of a 5 per cent gelatine gel, containing 0.01 N  $\text{K}_2\text{Cr}_2\text{O}_7$ , rings are soon formed. However, if the  $\text{AgNO}_3$  be enclosed in 5 per cent gelatine and placed on top, practically no ring formation occurs. Without doubt, if we could, by freezing or otherwise, regulate the rate or meeting, and of the reaction of the ions in water solution, to the same rate as in gelatine, rings would also be visible in the water. Dreaper's work which we have corroborated substantiates this opinion.

## SUMMARY.

A theory of rhythmic precipitation is advanced, which accounts for the action of the gel. This theory accepts the descriptions of the process given by Bradford and by Holmes, which are essentially alike. Precipitation in gels does not differ from precipitation in water; therefore, adsorption is no more important in gels where precipitation is banded, than in water where it is continuous. The function of the gel is to fix the precipitate where it is formed, and to restrict the zone of the influence of the ions, by reducing to a minimum the factors which make for turbulence or diffusion. It is pointed out that any influence that lessens the turbulence of the reaction, makes for rhythmic precipitation.

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## GROWTH ON DIETS HIGH IN CARBOHYDRATE AND HIGH IN FAT.

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The phenomenon of growth is a function of at least two factors. First, there is what might be called the heredity factor, the growth impulse, which is characteristic for each species. In the usual conception of growth this element may be considered to be structural in nature for it probably resides in and is transmitted through certain cellular elements. The second determinant of growth may be called the environment factor. In this category of influences must be classed the physical and chemical characteristics of the surrounding medium which in turn includes food materials and the respirable gases.

Over the heredity factor the experimentalist has attained only a slight degree of control. The environmental factor on the other hand is more amenable to study and experimental variation. It is only when both growth influences are at the optimum that ideal growth characteristic for the species is obtained. The science of nutrition concerns itself with the determination of the optimal environmental conditions under a large variety of circumstances.

The classical researches of Hopkins, of Osborne and Mendel, and of McCollum and his associates in the field of nutrition have emphasized the necessity of careful attention to details of the diet which are of greatest consequences in the problems of maintenance and growth. The appreciation of the importance of the character and relative abundance of the amino-acids in the protein fed, of the proper balance of the inorganic salts together with the indispensability of certain of them, and of the discovery and partial characterization of the vitamins, together with the far-reaching effects of the lack of these food

factors, have been outstanding contributions of the 20th Century to physiology. But the attraction of these more modern conceptions must not overshadow the fundamental consideration of the "quantity" idea in nutrition. The growing animal needs calories quite as much as it needs vitamins. The various factors must work hand in hand, for the absence of any one of the recognized dietary components means nutritive failure. However, the point of view must change with the passage of time, and often, rapidly. Discoveries of the past two decades have repeatedly afforded explanations for the nutritive failure of animals on synthetic diets which were considered adequate in only the last century. It is the privilege, therefore, of the investigator of to-day to reestablish many of the fundamental principles in the light of the more recent developments in the science.

The purpose of the present experiments is to determine the efficiency of calories from various sources in supplying the energy demand for growth when all the other factors in the diet are adequately provided to the best of our present knowledge. Will calories derived largely from carbohydrate equal in growth-promoting power those obtained largely from fat? The practical bearing of the answer to such a question in the live stock industry is far-reaching. A second point of more strictly academic interest involves the adjustment of the growing animal to calorie intake on diets of such widely different energy values as those herein discussed.

Comparisons of carbohydrate with fat have been made using various other criteria. On the basis of their relative efficiencies in protein-sparing action, carbohydrate appears to be superior to fat (Tallquist (1902), Cathcart (1922), and Kayser (1894)). Likewise, Krogh and Lindhart (1920) found that exercise can be more economically performed from the energy point of view when carbohydrate is being burned than when fat is undergoing oxidation. The inability of fats to provide the requisite energy for the physiological maintenance of the diabetic organism is too well known to need further comment. In the light of this and other evidence of similar nature, might we expect fat calories to function as efficiently as carbohydrate calories in growth, one of the most severe requirements made on the animal metabolism?

Until recently, attempts at feeding animals with diets free from fats have resulted in failure because of the unconscious omission of the fat-soluble vitamin in the foods and as a result false conclusions were drawn concerning the importance of natural fats in the diet. Equipped with the knowledge of the indispensability of vitamin A, Drummond (1920) used an alcohol extract of carrots as a source of this food factor with a diet containing only minute traces of ether-soluble substances. The young rats did not grow normally on this ration. Osborne and Mendel (1920) also studied the growth of rats on foods exceedingly low in fats, using 0.2 gm. of alfalfa as the source of the fat-soluble factor. Upon this high carbohydrate diet normal growth from about 75 to approximately 280 gm. was obtained in most of the animals. Growth experiments<sup>1</sup> have been tried on diets high in fat; the animals grew normally for a time and then merely maintained themselves at a stationary weight. In this case the protein was fed at the same level as in the standard balanced diet, namely 18 per cent, with the result that, due to the diminished intake of the high calorie fat food, protein hunger probably became a factor in the inhibition of growth.

*Methods Employed.*—In the present investigation the effort was made to obtain the largest practicable part of the calories from either the fat or the carbohydrate. The diets were carefully planned so that, no matter what the caloric value of the food, the ratio of protein calories to total calories was the same as in the standard balanced ration. In addition, the ratio of salt content to total calories was also constant in all the diets. The adjustment of intake to caloric value of the food could therefore be made by the rats without disturbing the actual consumption of the two indispensable constant factors; namely, the inorganic salts and the protein. Since the protein used was casein, fed at a safe level (Osborne and Mendel, 1916), and vitamins A and B were adequately supplied, the diets were complete for growth of rats as far as we know at the present time.

The "balanced" control, high fat, and high carbohydrate diets had the following compositions.

<sup>1</sup> The results of unpublished experiments of Osborne and Mendel personally communicated by Dr Mendel

	Amount	Cal. per kg. food	Apportionment of total calories.
Balanced control diet.			
	<i>per cent</i>		<i>per cent</i>
Casein* . . . . .	18	738	Protein . . . . . 13.8
Starch . . . . .	51	2,091	Carbohydrate. . . 39.2
Lard . . . . .	23		
Cod liver oil . . .	4	2,511	Fat.. . . . . 47.0
Salts† . . . . .	4		
Total . . . . .	100	5,340	100.0

## High fat diet.

Casein	25	1,025	Protein . . . 13.8
Lard .	65		
Cod liver oil . . .	4	6,417	Fat . . . . 86.2
Salts . . . . .	6		
Total . . . . .	100	7,442	100.0

## High carbohydrate diet

Casein	14	574	Protein . . . 13.8
Starch	79	3,239	Carbohydrate . . 77.8
Cod liver oil	3.8	353	Fat . . . . . 8.4
Salts . . . . .	3.2		
Total . . . . .	100.0	4,166	100.0

\* A "fat-free" product from the Casein Company, 13 Park Row, New York City. It was washed with water of pH 4.6 seven times, then with alcohol (95 per cent) and ether, and dried in a current of warm air.

† The inorganic salt mixture described by Osborne and Mendel (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557).

The carbohydrate food was made up as a thick, smooth starch paste with the weighed amount of starch. This was allowed to cool and the other ingredients stirred into it. The moist food was then spread in thin layers in pans and dried to a crisp cracker at 60–70°. The starch was partially dextrinized by this treatment.

In all the experiments vitamin B was provided by 30 mg.<sup>2</sup> of "Yeast Vitamine-Harris," fed apart from the food, while the

<sup>2</sup> This dose had previously been found adequate for growth over the range involved in these experiments.

fat-soluble factor was carried by the cod liver oil in the food mixtures. The technique employed was similar to that outlined by Ferry (1920).

*Results.*—A comparison of the actual age-weight curves showed that the best growth rate was obtained on the "high carbohydrate" diet while on the "high fat" diet, growth was distinctly subnormal in all cases. Although in the present series of experiments the rats on the "balanced" control diet grew slightly below normal in some cases, we have many instances of growth

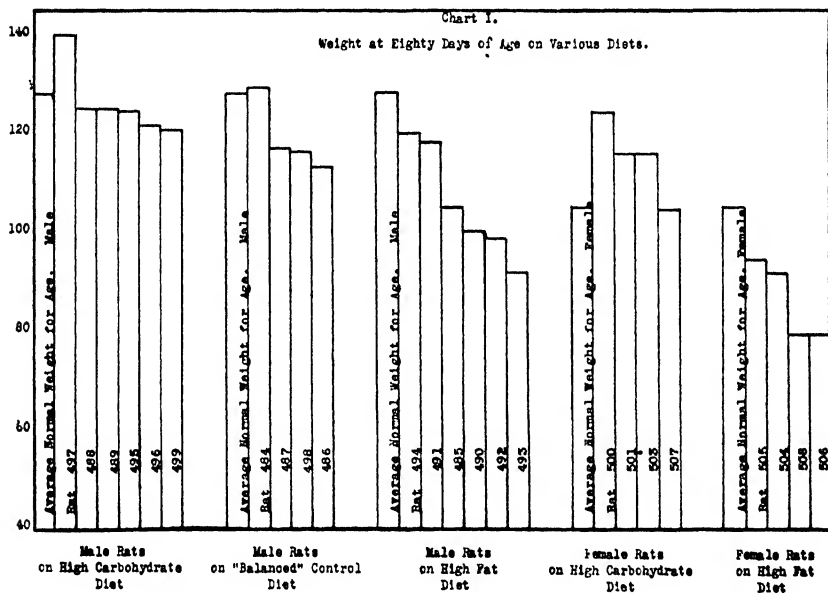


CHART I.

better than normal on this diet. Chart I shows diagrammatically the weight attained by the various groups at a certain age, the superiority of both male and female rats on "high carbohydrate" diet being very apparent.

On the "high carbohydrate" diet the males grew at the normal rate for the white rat<sup>3</sup> while the females grew somewhat better after about 70 days of age.

<sup>3</sup> These are data of Osborne and Mendel taken from Donaldson (Donaldson, H. H., *The rat*, Philadelphia, 1915).



The growth rate of the male rats on the "high fat" diet began diminishing at about 50 days of age and thereafter they grew at a distinctly subnormal rate. On the other hand, the females on the "high fat" diet grew at a rate approximating the normal somewhat more closely. These results indicate that under comparable conditions, and with all the other food factors being identical, rats obtaining the larger part of their calories from fat grow less rapidly than either those using largely carbohydrate calories or those obtaining energy from a balanced diet.

The *appearance* of the two groups of animals was distinctly different. Those on the fat food were sleek and plump though obviously small while those on the carbohydrate food were larger and more rangy though with good coats.

TABLE I  
*Average Weekly Food Intake*

Age	Males on fat food	Males on carbohydrate food	Males on standard food	Females on fat food	Females on carbohydrate food
<i>days</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
50	14	27	24	21 5	51
70	28 5	57	47	19	53 5
90	28	59 5	45	21	56
110	26	60	47 5	26	65
130	30	73	51	32	55
150	34		50		

In Table I is given the average weekly gross food intake on the various diets. The rats referred to in the table were not making comparable gains and so the figures can only show roughly the adjustment to calories in the food. The 73 gm. eaten per week by the rats on "high carbohydrate" diet can be compared with the 51 gm. of standard food eaten by rats of the same age growing at a like rate. The figures are comparable to those quoted by Osborne and Mendel for rats twice as old. As might be expected *a priori*, the intake of "high fat" food was smaller than that of either of the other diets.

In order to obtain more accurate figures as to the adjustment to calories, food intake on the various diets was compared over like periods when normal growth was obtained. In Table II are shown the total calories consumed by male rats to gain 21 gm. between 40 to 50 days of age; *i.e.*, growing at the normal rate.

This range on the growth curve was chosen because after 50 days of age the male rats on the "high fat" diet ceased to grow at the normal rate.

In Table III the female rats are compared during the period 40 to 50 days of age while growing at the normal rate; *i.e.*, gaining 19 gm.

From the tabulated data, it is obvious that when growing at the normal rate on diets qualitatively and quantitatively adequate, young rats adjust to approximately the same caloric intake whether the major part of the calories are obtained from fat or carbohydrate or from a mixture of the two. The agree-

TABLE II.  
*Food Intake for Males.*

Diet	Average gm food to gain 21 gm body weight	Caloric value per gm	Average total calories.
Standard . . . . .	58 0	5 3	307
High fat . . . . .	41 5	7 4	305
" carbohydrate . . . . .	82 0	4 1	336

TABLE III  
*Food Intake for Females*

Diet	Average gm food to gain 19 gm body weight	Caloric value per gm.	Average total calories.
High fat . . . . .	30 5	7 4	226
" carbohydrate . . . . .	66 5	4 1	273

ment is more striking between the standard and the "high fat" diets than between these and the "high carbohydrate food." Rubner's law of the "Constant energy expenditure" states that for certain animals comparable growth is made at the expense of comparable calories absorbed, whether the growth is fast or slow. Hopkins (1912) has showed that this generality applies to the rat on a diet similar to our standard "balanced" ration. There must be, therefore, a decided wasting of energy in the growth metabolism of this animal on high carbohydrate diet, for in this case 10 per cent more food was consumed than on either of the other diets during the period of comparable growth. At whatever age they

are compared, the high carbohydrate-fed rats consumed more calories than the standard diet rats growing at the same rate.

#### DISCUSSION.

The failure of the rats on the "high fat" diet to maintain their initial normal growth rate appears to be due, partly at least, to the lack of desire for the food, for after growth became subnormal the calorie intake was definitely smaller than that of the rats on standard food, whereas before, it had been the same. It has been our experience, in confirmation of that of others, that appetite may be restored by adding any of the recognized vitamins to diets lacking these food factors. Likewise, appetite that has failed absolutely on a protein-free, though otherwise adequate diet, is restored by replacing the protein.<sup>4</sup> In the present experiment in an effort to increase the appetite, the yeast vitamin powder was increased from 30 to 50 mg. per day in the case of four rats on the "high fat" food, but without definite improvement in the growth rate. Substituting a food containing only 59 per cent of fat and 11.9 per cent of carbohydrate for the original "high fat" food (69 per cent fat) for Rat 493, resulted in an augmented food intake and in an immediate response in normal growth. Landergren (1903) has stated that an "undetermined minimum" of carbohydrate, whether preformed in the food or stored in the organism as glycogen, can serve to diminish a previously existing negative nitrogen balance on a carbohydrate-free food. A similar principle seemed to operate in these experiments. The possibility of affecting appetite and thus, growth, by adjusting the fat and carbohydrate in an otherwise adequate diet is a new conception in nutrition and is worthy of more extensive experimental study. It is more than likely that, in spite of the highly emphasized potencies of newly discovered food factors, normal nutritive well-being will be secured only when the proper ratio, as well as the adequate absolute amounts of all of the recognized dietary principles, is maintained.

It is known that the normal organism (human) on diets containing large amounts of fat and little or no carbohydrate, excretes acetone bodies in the urine. Is it not probable, there-

<sup>4</sup> Unpublished data from this laboratory.

fore, ~~the~~ in addition to a diminished intake due to lack of appetite, the young animal is losing energy through the imperfect combustion of the fatty acids, the end-result being manifested in a subnormal growth rate?

In these experiments it is shown that within rather narrow limits the consumption of food is adjusted to the caloric value of that food. From the point of view of the energy value of food, the animal may be said literally to "eat calories." It is desirable, however, to study this point further in the case of the rats on the high fat rations when they can be made to eat enough of the food to grow at the normal rate.

#### SUMMARY.

Rats making comparable increments of growth in the same time consume nearly the same number of calories from diets high in fat, high in carbohydrate, or from intermediate "balanced" diets.

Rats on the high carbohydrate food grew normally throughout the period of observation (151 days), but animals on the high fat ration failed to maintain the normal growth rate after 50 days.

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## STUDIES WITH THE FOLIN AND WU BLOOD SUGAR DETERMINATION.

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Among the several methods for quantitative blood sugar estimations satisfactory from the clinical point of view, that of Folin and Wu, presented with their system of blood analysis (1) and subsequently modified (2), recommends itself highly and justly enjoys widespread popularity. One precaution which the authors mention as necessary, namely the use of a standard solution which contains approximately the same amount of dextrose as the blood filtrate being tested, does not appear to us to have been sufficiently emphasized. Folin and Wu recommend the use of one standard containing 0.1 mg. of dextrose per cc. of water, and another containing 0.2 mg. of dextrose per cc. of water. After the dilution of the blood ten times, as occurs in precipitating the proteins according to Folin's technique (1), the filtrate from a blood containing 100 mg. of dextrose per 100 cc., and from one containing 200 mg. per 100 cc., contains the same amount of dextrose as the 0.1 and 0.2 mg. standards, respectively. These standard dextrose solutions are to be used, except in unusual instances, for comparison in all blood sugar determinations, certainly for all those on bloods containing between 100 and 200 mg. of dextrose per 100 cc. In our hands these two standard solutions are not sufficient. If the filtrate from a blood containing 150 mg. of dextrose per 100 cc. of blood is read against each of these standards, the results may differ more than 15 per cent, the reading against the 100 mg. standard having a plus error, and that against the 200 mg. standard having a minus error. For this reason we have in the past regularly used three standard dextrose solutions, the equivalents of 100, 150, and 200 mg. of dextrose per 100 cc. of blood; and occasionally even more standards than these. Systematic experi-

ments to investigate this point prove the necessity for using more than two standards if accurate results are to be obtained on all bloods with the Folin and Wu method. This paper is a report of these experiments which give added emphasis to the statement of these authors that with this method the standard solution must contain approximately the same amount of dextrose as the protein-free blood filtrate being tested.

Experiments were carried out with a specimen of dextrose on the laboratory shelves not specially identified, with Kahlbaum's glucose, and with Difco dextrose. The results obtained were essentially the same with each of the sugars used. Different strengths of solutions were prepared from these sugars, and the Folin and Wu blood sugar determination was carried out on each at the same time and compared to the standard dextrose solutions equivalent to 100 and to 200 mg. of dextrose per 100 cc. of blood. Some of these observations were repeated by three different workers, and many were carried out by two. Readings were made on four colorimeters of different make. The sample experiments reported in this paper were done with Difco dextrose which had been polarized and found to be pure, and the readings were made on a Bausch and Lomb plunger colorimeter.

*Experiment 1*—Dextrose solutions of different strengths were read against a standard solution containing 0.1 mg. of dextrose per cc. of water, the equivalent of 100 mg. of dextrose per 100 cc. of blood. The dextrose solutions were made up as follows: 0.06, 0.07, 0.08 mg., etc., up to 0.2 mg. of dextrose per cc. of water, the equivalent of 60, 70, 80, etc., up to 200 mg. of dextrose per 100 cc. of blood.

The routine Folin and Wu blood sugar determination was carried out on each of these solutions in duplicate at the same time, using 2 cc., exactly as is done with protein-free blood filtrate. Readings were made against the 0.1 mg. standard set at 20 on the colorimeter scale on one determination of each strength of dextrose solution; the standard solution was then read against itself again, and readings were made on the duplicate determination of each strength of dextrose solution. All the readings were completed within a half hour of the time the blue color had been developed after the addition of the phosphate-molybdate sugar reagent. This experiment was repeated on the following day with freshly prepared dextrose solutions. The four values for each strength of dextrose solution thus obtained checked up very closely. The average of the four readings appears in Table I.

*Experiment 2*—Dextrose solutions of different strengths were read against a standard solution containing 0.2 mg. of dextrose per cc. of water, the equivalent of 200 mg. of dextrose per 100 cc. of blood. The dextrose

TABLE I.

*Various Strengths of Dextrose Solution Read Against a Standard Dextrose Solution Equivalent to 100 Mg. of Dextrose per 100 Cc. of Blood, Set at 20 on the Colorimeter Scale.*

Equivalent in dextrose per 100 cc blood, of dextrose solutions tested.	Correct reading on colorimeter for ratio concerned	Average of colorimeter readings obtained	Dextrose calculated from colorimeter readings	Error of dextrose	Error
<i>mg.</i>			<i>mg</i>	<i>mg</i>	<i>per cent</i>
200	10	7 9	253 2	+53 2	+26 6
175	11 43	9 6	208 3	+33 3	+19 02
150	13 33	11 8	169 6	+19 6	+13 06
140	14 29	12 9	155 0	+15 0	+10 71
130	15 38	14 2	140 8	+10 8	+8 3
120	16 66	15 8	126 6	+6 6	+5 5
110	18 18	17 7	113 0	+3 0	+2 72
100	20 0	20 0	100 0	0	0
90	22 22	23 2	86 3	-3 7	-4 11
80	25 0	26 8	74 7	-5 3	-6 63
70	28 57	32 1	62 3	-7 7	-11 0
60	33 33	40 2	49 8	-10 2	-17 0

TABLE II

*Various Strengths of Dextrose Solution Read Against a Standard Dextrose Solution Equivalent to 200 Mg. of Dextrose per 100 Cc. of Blood, Set at 10 on the Colorimeter Scale*

Equivalent in dextrose per 100 cc blood, of dextrose solutions tested	Correct reading on colorimeter for ratio concerned	Average of colorimeter readings obtained	Dextrose calculated from colorimeter readings	Error of dextrose	Error
<i>mg.</i>			<i>mg</i>	<i>mg.</i>	<i>per cent</i>
400	5 0	4 13	484 2	+84 2	+21 05
375	5 33	4 45	449 4	+74 4	+19 89
350	5 7	5 0	400 0	+50 0	+14 28
325	6 2	5 35	373 8	+48 8	+15 01
300	6 66	5 93	337 2	+37 2	+12 4
275	7 2	6 75	296 3	+21 3	+7 74
250	8 0	7 5	266 6	+16 6	+6 64
225	8 88	8 55	233 9	+8 9	+3 95
200	10 0	10 0	200 0	0	0
175	11 43	11 98	166 1	-8 9	-5 08
150	13 33	14 15	141 3	-8 7	-5 8
125	16 0	17 63	113 4	-11 6	-9 28



solutions were made up as follows: 0.125, 0.15, 0.175, etc., up to 0.4 mg. of dextrose per cc. of water, the equivalent of 125, 150, 175, etc., up to 400 mg. of dextrose per 100 cc. of blood. Exactly the same procedure outlined for Experiment 1 was carried out with these solutions except that the readings were made against this stronger standard, 0.2 mg. of dextrose per cc. of water, set at 10, instead of at 20, on the colorimeter scale. As in Experiment 1, the four values obtained for each strength of dextrose solution by carrying out determinations in duplicate on a series of such solutions on successive days, checked up very closely. The average of the four readings appears in Table II.

The readings, obtained in the experiments reported above where Folin and Wu blood sugar determinations were carried out on dextrose solutions of different strengths and compared with dextrose solutions equivalent to 100 and 200 mg. of dextrose per 100 cc. of blood, demonstrate an error too great to be ignored. Other experiments, similar to those recorded and some differing from them in minor points, permit only the same conclusion.

All the experiments reported above with solutions of dextrose in water were also carried out on blood filtrates. Specimens with the different sugar contents were obtained by diluting with water an ordinary protein-free blood filtrate in some instances, and in others by adding various amounts of dextrose to the water used for dilution of the blood preparatory to precipitation of the proteins. The findings of different experiments with blood filtrates did not parallel each other so closely as when carried out with aqueous solutions of dextrose, but they were all essentially the same and compelled the same conclusions. Furthermore, many routine blood sugar determinations read against the two standard solutions recommended by Folin and Wu also demonstrated that for accurate results with this method, the standard solution used must contain very nearly the same amount of dextrose as the blood filtrate being tested.

The outstanding possibilities as to the source of the error in the Folin and Wu blood sugar determination which suggest themselves are:

1. The amount of reduction by more concentrated solutions of dextrose is possibly not proportional to that by weaker solutions.
2. The amount of reoxidation of the reduced copper and, therefore, development of color by the added phosphomolybdate-phosphotungstate sugar reagent is perhaps not proportional in sugar solutions of different strengths.

3. The curve of color dilution of the color developed in this test may be irregular; in other words, it may not follow Beer's law which states that in colorimetric work the effect of doubling the concentration of a colored solution is the same as doubling

TABLE III

*Comparison of the Readings Obtained from Dextrose Solutions Read Against a Standard One-Half as Strong, with Those Obtained after Dilution of the Same Solutions to the Strength of the Standard.*

Tube No			Colorimeter reading obtained	Correct reading for ratio concerned
1	Used as standard against which all tubes that follow were read	Standard dextrose solution equivalent to 100 mg per 100 cc blood, diluted to 25 cc. mark, read against itself.	20 0	20 0
2	Duplicate of Tube 1	Standard dextrose solution equivalent to 100 mg per 100 cc blood, diluted to 25 cc mark, read against Tube 1	20 0	20 0
3		Standard dextrose solution equivalent to 100 mg per 100 cc blood, diluted to 12.5 cc mark, read against Tube 1	8 3	10 0
4		Standard dextrose solution equivalent to 200 mg. per 100 cc blood, diluted to 25 cc. mark, read against Tube 1	8 2	10 0
5		Tube 4 diluted with equal portions of water; read against Tube 1	20 0	20 0

Variations from 20 of less than two-tenths marks on the Vernier scale have been disregarded in this table

the depth of the column through which it is viewed. The following experiment, carried out with all the same solutions used in the experiments above, sheds light on these points.

*Experiment 3.*—The routine Folin and Wu blood sugar determination was carried out at the same time on three tubes containing 2 cc. of a 0.01 per cent dextrose solution, the standard equivalent to 100 mg. of dextrose per 100 cc. of blood, and on one tube of a 0.02 per cent solution, the standard equivalent to 200 mg. of dextrose per 100 cc. of blood. Two tubes of the 100 mg. standard were diluted to the 25 cc. mark, and one to a 12.5 cc. mark on the Folin and Wu blood sugar tube. The 200 mg. standard tube was diluted to the 25 cc. mark, mixed, and from this solution 5 cc. were taken and diluted immediately with an equal quantity of water. All these tubes were then read against one of the 100 mg. standard tubes, diluted to the 25 cc. mark, set at 20 on the colorimeter scale. The readings appear in Table III.

The findings in Experiment 3 demonstrate:

1. That a dextrose solution, which differs much in strength from that of the standard against which it is read, will give readings which vary widely from those which are correct for the ratios concerned.

2. That such a dextrose solution, when diluted to the same strength as the standard after reduction and development of the color, will give an absolutely correct reading.

It is interesting that the 0.01 per cent dextrose solution when diluted to 12.5 cc. and compared with itself diluted to 25 cc., set at 20 on the colorimeter scale, read a little above 8 instead of 10; that the 0.02 per cent dextrose solution diluted to 25 cc. read against the same standard did not read 10, but also read a little above 8, essentially the same as the 0.01 per cent dextrose solution diluted to 12.5 cc.; and that a portion of this 0.02 per cent dextrose solution, diluted with equal portions of water, read not twice as much as the undiluted portion—or between 16 and 17—but compared exactly with the 0.01 per cent solution diluted to 25 cc. This would indicate that the colorimeter readings differing from those which are correct for the ratio concerned, which were made in Experiments 1 and 2, were not due to the first two possible sources mentioned, that is to differences in the rate of reduction of copper in the test or in the development of the color obtained, but rather to the irregularity of the curve of color dilution of the blue color dealt with.

The results recorded which justify this conclusion are only obtained when the instructions of Folin and Wu are followed closely. Other sources of error are introduced; for example, if the dilution after development of color is not carried out prop-

erly, and if inferior reagents are used. In regard to the latter, it is important to have a good phosphomolybdate-phosphotungstate sugar reagent. With an inferior sugar reagent, an additional error, apparently due to a disproportionate reoxidation of the reduced copper solution, is introduced. With water-clear sugar reagents, as insisted upon by Folin and Wu, this error is not introduced. If the sugar reagent has a tinge of yellow, this error may not be encountered, but it often is. Studies with sugar reagents are now in progress and will be reported later. Suffice here to say that the chemicals employed in its preparation are more important than minor variations in technique and that we have obtained most satisfactory results with Baker's molybdic acid and Merck's phosphoric acid.

In a recent paper by Morgulis, Edwards, and Leggett (3), the arsenotungstate solution of Benedict for uric acid determinations (4) is modified and used instead of the sugar reagent of Folin and Wu. This new reagent develops more color than the sugar reagent of Folin and Wu, and it was hoped that thereby the error due to the irregularity of the curve of color dilution might be eliminated. In our hands this was found not to be the case; and indeed it was hardly to be expected because the authors of this revised method report results with it which checked those obtained by the Folin and Wu method.

#### SUMMARY.

When Folin and Wu blood sugar determinations are carried out on dextrose solutions of different strengths and on blood filtrates containing different amounts of dextrose, all except those containing approximately the same amount of dextrose as the standard used yield readings on the colorimeter differing from those which are correct for the ratio concerned, too far to be negligible. If, for example, a dextrose solution containing 15 mg. per 100 cc. of water, or the filtrate from a blood containing 150 mg. of dextrose per 100 cc., is read against both the standard solution equivalent to 100 mg. and that equivalent to 200 mg. of dextrose per 100 cc. of blood, the readings obtained are such that the final calculated results may differ from each other more than 15 per cent. These observations add further emphasis to the statement of Folin and Wu that in their quantitative blood sugar deter-

mination, the standard solution should contain very nearly the same amount of dextrose as the blood filtrate being tested. If this test on most blood filtrates is never read against any standard but that equivalent to 100 mg., or that equivalent to 200 mg. of dextrose per 100 cc. of blood, a large error will result in many instances.

Several sources of error may be demonstrated, but if the instructions of Folin and Wu are followed closely, only that due to the irregularity of the curve of color dilution of the blue color being dealt with, is of sufficient magnitude to be of any importance.

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## A MODIFIED FOLIN AND WU BLOOD SUGAR METHOD.

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In the preceding paper data have been presented to emphasize anew the statement of Folin and Wu that in their method for quantitative blood sugar estimation<sup>1</sup> the standard solution must contain very nearly the same amount of dextrose as the blood filtrate being tested. Examination of these data shows that if approximately correct results on all blood filtrates are to be obtained with this method it would be necessary to have more standard solutions than can be used conveniently. This is necessary, probably in large part although perhaps not entirely, because the curve of color dilution of the color developed in this procedure is irregular and, therefore, if the standard and unknown are not almost the same color at the time the colorimetric readings are made, errors are introduced. An error in a method used for quantitative work is unfortunate under any circumstances, and particularly so in a blood sugar method of widespread popularity in these days of insulin therapy of diabetes when correct values for the blood sugar, especially when low, are so important. In this report a minor modification of the Folin and Wu method for blood sugar determination is presented whereby the color developed by the unknown blood filtrate is always the same as that of the standard dextrose solution at the time the colorimeter readings are made; and one standard alone may be used for comparison with many blood filtrates and two, or at most three standards, will make possible correct determinations of the amount of dextrose in any blood filtrate ever encountered.

The modification of the Folin and Wu method for quantitative blood sugar determination used in this laboratory consists merely in: (1) Diluting the unknown after the addition of the phos-

<sup>1</sup> Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.

phomolybdate-phosphotungstate sugar reagent and development of color until it is approximately the same shade as the standard, instead of to a constant amount, before colorimetric comparison is made; and (2) introducing this variable factor, the amount of dilution, in the final formula for calculating the amount of dextrose in the unknown. The determination is carried out in tubes

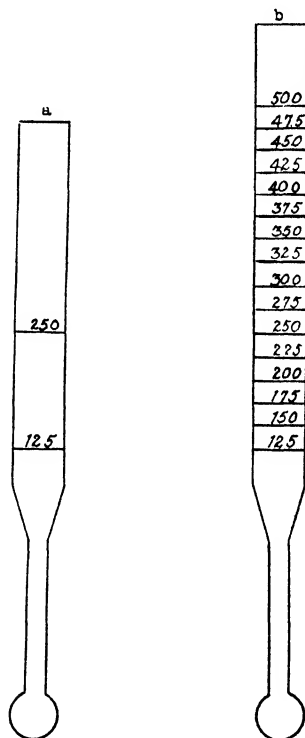


FIG. 1. a, Tube used for the Folin and Wu blood sugar determination. b, Tube used for the modified Folin and Wu blood sugar determination.

differing from those suggested by Folin and Wu<sup>1</sup> only in that they are longer and graduated in steps of 2.5 cc. from 12.5 to 50.0 cc., Fig. 1.<sup>2</sup> In every other particular the Folin and Wu technique is followed.

<sup>1</sup> Satisfactory tubes of this character may be purchased from The Emil Greiner Company, New York.

This modification will be practical only if the error in the Folin and Wu method for estimating blood sugar such as was demonstrated in Tables I and II of the preceding paper was due entirely, or almost entirely, to irregularity in the curve of color dilution of the color developed in the determination. Experiments presented in the preceding paper demonstrate that this is so, provided the instructions of Folin and Wu are carefully followed. Their statement that the phosphomolybdate-phosphotungstate sugar reagent must be water-clear is especially important, because if it has a yellowish tinge an additional source of error may be introduced. Other errors may also be present; for example, the amount of reduction by more concentrated solutions of dextrose is possibly not proportional to that by weaker solutions; the degree of re-oxidation which perhaps takes place in performing the determination may not be the same for strong and weak solutions of dextrose; the amount of water added after development of color may introduce an error; and several other features must be considered. If these errors are present, they are so small that they are within the limits of error for ordinary colorimetric determinations, so that if that due to the use of inferior reagents is eliminated, the only error of consequence remaining is that due to the irregularity of the curve of color dilution of the color being dealt with. Before the modification herein suggested was adopted for routine use, however, several experiments were carried out to prove that it was practical.

*Experiment 1.*—A specimen of Difco dextrose shown to be pure by polariscopic examination was used to make dextrose solutions of the following strengths 0.11, 0.12, 0.13, etc., up to 0.2 mg. of dextrose per cc. of water, the equivalent of 110, 120, 130, etc., up to 200 mg. of dextrose per 100 cc. of blood. The routine Folin and Wu blood sugar determination was carried out on each of these solutions, using 2 cc., exactly as is done with the protein-free blood filtrate. At the same time the procedure was carried out with these solutions in the new tubes described above, but diluted, after the addition of the phosphomolybdate-phosphotungstate sugar reagent and development of color, to the amount at which theoretically they would compare exactly in color with the standard containing 0.1 mg. of dextrose per cc. diluted, after the addition of the reagent and development of color, to 25 cc. All these dilutions were made promptly, at as nearly the same time as possible. Each test was compared in the colorimeter with a standard dextrose solution containing 0.1 mg. of dextrose per cc., the same amount as is in the filtrate from a blood containing 100 mg. of dextrose per 100 cc., set at 20 on the colorimeter scale. The results appear in Table I.



*Experiment 2*—Dextrose solutions of the following strengths were prepared: 0.22, 0.24, 0.26, etc., up to 0.4 mg. of dextrose per cc. of water, the equivalent of 220, 240, 260, etc., up to 400 mg. of dextrose per 100 cc. of blood. Procedures similar to those outlined for Experiment 1 were carried out on these dextrose solutions and the comparison in the colorimeter was made against a standard dextrose solution containing 0.2 mg. of dextrose per cc., the same amount as is in the filtrate from a blood containing 200 mg. of dextrose per 100 cc., set at 10 on the colorimeter scale. The results appear in Table II.

TABLE I

Equivalent in dextrose per 100 cc blood of dextrose solutions tested	Determinations according to original Folin and Wu method Contents of tubes diluted to a volume of 25 cc			Determinations according to the modified method Contents of tubes diluted to volume at which the color of the unknown should match, theoretically, that of standard			
	Average of colorimeter readings.	Dextrose recovered per 100 cc.	Error	Volume to which contents of tubes were diluted.	Average of colorimeter readings	Dextrose recovered per 100 cc	Error.
	Solutions read against the 100 mg standard set at 20 on the colorimeter scale			Solutions read against the 100 mg standard set at 20 on the colorimeter scale			
mg		mg.	per cent	cc.		mg.	per cent
100	20 0	100 0	0	25 0	20 0	100 0	0
110	17 6	113 6	3 3	27 5	19 9	110 5	0 5
120	15 6	128 2	6 8	30 0	19 9	120 6	0 5
130	14 2	140 8	8 3	32 5	19 9	130 7	0 5
140	12 6	158 7	13 3	35 0	20 0	140 0	0
150	11 7	170 9	13 9	37 5	19 9	150 8	0 5
	Solutions read against the 200 mg standard set at 10 on the colorimeter scale						
160	13 8	144 9	9 4	40 0	19 9	160 8	0 5
170	12 4	161 3	5 1	42 5	19 8	171 7	1 0
180	11 7	171 3	4 8	45 0	19 9	180 9	0 5
190	10 7	186 9	1 6	47 5	19 9	191 0	0 5
200	10 0	200 0	0	50 0	19 9	201 0	0 5

Because of the results obtained in Experiments 1 and 2 we expected to find that one standard solution would suffice for determinations on all blood filtrates; that the color developed by the test on the blood filtrate from a blood containing 400 mg. of dextrose per 100 cc. when diluted to 50 cc. would match that developed by the standard dextrose solution equivalent to the filtrate from a blood containing 100 mg. of dextrose per 100 cc., when diluted to 12.5 cc. In other words, we expected to find

that the color developed by a dextrose solution four times as strong as the standard solution, when diluted to 50 cc., would match that of the standard solution diluted to 12.5 cc. Such, however, was found not to be the case. Under these conditions a plus error of about 8 per cent is introduced so that the color developed by the stronger solution must be diluted to about 55 cc. before it matches that of the 100 mg. standard diluted to 12.5 cc. Apparently, therefore, the possible sources of error mentioned above, other than the irregularity of the curve of color

TABLE II.

Equivalent in dextrose per 100 cc. blood of dextrose solutions tested	Determinations according to original Folin and Wu method Contents of tubes diluted to a volume of 25 cc			Determinations according to the modified method Contents of tubes diluted to volume at which the color of the unknown should match, theoretically, that of standard			
	Average of colorimeter readings	Dextrose recovered per 100 cc	Error	Volume to which contents of tubes were diluted	Average of colorimeter readings	Dextrose recovered per 100 cc	Error
	Solutions read against the 200 mg. standard set at 10 on the colorimeter scale			Solutions read against the 200 mg. standard set at 10 on the colorimeter scale			
mg		mg	per cent	cc		mg	per cent
200	10 0	200 0	0	25 0	10 0	200 0	0
220	8 6	232 6	5 7	27 5	10 0	220 0	0
240	7 7	259 7	8 2	30 0	9 9	242 4	1
260	7 0	285 7	9 9	32 5	9 9	262 6	1
280	6 0	333 3	19 0	35 0	10 0	280 0	0
300	5 6	357 1	19 0	37 5	10 0	300 0	0
320	5 7	350 9	9 7	40 0	9 9	323 2	1
340	5 1	392 1	15 3	42 5	9 9	343 4	1
360	4 9	408 1	13 4	45 0	9 9	363 6	1
380	4 3	465 1	22 4	47 5	9 8	387 7	2
400	4 1	487 8	21 9	50 0	10 0	400.0	0

dilution of the color developed, do come into play. When the unknown does not differ more than 100 per cent from the standard, however, the error introduced by these factors is too small to be demonstrated by ordinary colorimetric determinations, but when there is a difference much greater than 100 per cent an appreciable error creeps in. When the standard dextrose solution equivalent to the filtrate from a blood containing 100 mg. of dextrose per 100 cc. is used for comparison by this revised method with the

filtrate from a blood containing 400 mg. of dextrose per 100 cc. the error is less than when the determination on a similar filtrate diluted to 25 cc. is read against a 200 mg. standard, diluted to the same amount as ordinarily done with the method of Folin and Wu. And, inasmuch as absolute values for the sugar of the blood when in such great concentration are not necessary in routine clinical work, one standard may be used with this revised method for all blood filtrates and fairly satisfactory results be obtained. If results closely approaching absolute values for all blood filtrates are desired, however, standard solutions differing from the sugar concentration in the unknown not more than 100 per cent must be used.

To perform the test according to the modification above suggested the Folin and Wu blood sugar determination on the standard dextrose solution (or solutions) chosen and on the unknown blood filtrates is carried out in tubes described above. After the addition of the phosphomolybdate-phosphotungstate sugar reagent and development of color, the test on the standard solution is diluted to 25 cc. and those on the unknowns are diluted to the cubic centimeter mark on the tube at which the color intensity most closely resembles that of the standard. The amount of dilution of each unknown is recorded and they are then compared in the colorimeter with the standard preparation. If the dilutions of the unknowns in the tubes have been made carefully, they should read on the colorimeter scale very nearly that figure at which the standard preparation was set.

When, as here obtains, the dilution of the unknown is not the same as the standard solution, the formula presented by Folin and Wu for calculating the final results by their method, namely

$$\frac{20}{\text{reading}} \times 100 = \text{mg. of dextrose per 100 cc. of blood,}$$

must be multiplied by the fraction

$$\frac{\text{dilution of unknown}}{\text{dilution of standard (25 cc.)}}$$

We have, therefore,

$$\frac{20}{\text{reading}} \times 100 \times \frac{\text{dilution of unknown}}{25}$$

or simplifying,

$$\frac{80 \times \text{dilution of unknown}}{\text{reading}} = \text{mg. of dextrose per 100 cc. of blood.}$$

If the amount of dilution necessary to make the color developed by the unknown blood filtrates match that of the standard is considered in relation to the strength and dilution of the standard used, a rough idea of the amount of dextrose in the bloods in question may be obtained. For instance, if the standard dextrose solution, equivalent to the filtrate from a blood containing 100 mg. of dextrose per 100 cc., diluted to 25 cc., is used, the values are as follows:

Dilution of unknown solution	Dextrose per 100 cc of blood	Dilution of unknown solution	Dextrose per 100 cc. of blood
cc	mg	cc	mg.
12 5	50	32 5	130
15 0	60	35 0	140
17 5	70	37 5	150
20 0	80	40 0	160
22 5	90	42 5	170
25 0	100	45 0	180
27 5	110	47 5	190
30 0	120	50 0	200

If the standard dextrose solution, equivalent to the filtrate from a blood containing 200 mg. of dextrose per 100 cc., diluted to 25 cc., is used, the values are as follows:

Dilution of unknown solution	Dextrose per 100 cc of blood	Dilution of unknown solution	Dextrose per 100 cc. of blood
cc	mg	cc	mg
12 5	100	32 5	260
15 0	120	35 0	280
17 5	140	37 5	300
20 0	160	40 0	320
22 5	180	42 5	340
25 0	200	45 0	360
27 5	220	47 5	380
30 0	240	50 0	400

Folin and Wu point out clearly that in their method for blood sugar determinations it is necessary, after the addition of the

phosphomolybdate-phosphotungstate sugar reagent and development of the color, to carry out the subsequent dilution on the standard solution and on the unknown blood filtrate at the same time. Experiments in this laboratory demonstrated the importance of this instruction, for if the dilutions of the standard and unknown are made 5 minutes apart an appreciable error is introduced. This observation is of importance in relation to the modification of the Folin and Wu blood sugar method here presented, because the dilution of the test on the unknown blood filtrate is not made all at once, but in two, or more, steps. If one keeps in mind the necessity for dilution of the standard and unknown at approximately the same time, and works rapidly, it is possible to dilute at least six unknowns to match the standard promptly enough to eliminate this source of error. The method of procedure which has been devised with this point in mind is as follows: After the addition of the phosphomolybdate-phosphotungstate sugar reagent and development of color—but before dilution—the tests on unknown blood filtrates are compared with that on the standard dextrose solution. One can tell whether the color developed in the unknowns is less than, about the same as, or more than that of the standard dextrose solution; and depending on this observation the unknowns are immediately diluted to a mark of less than 25 cc. to the 25 cc. mark, or to a mark of more than 25 cc. The standard is then diluted to 25 cc., the contents of each tube are thoroughly mixed, and they are compared again. Any further dilution of the tests on the unknown blood filtrates which are necessary to make them about the same color as the standard is carried out promptly. If it is expected that any of the protein-free filtrates being tested are from bloods containing more than 200 mg. of dextrose per 100 cc., it is wise to carry out at the same time a preparation not only on a standard dextrose solution containing 0.1 mg. per cc. of solution, but also on one containing 0.2 mg. per cc. of solution; for, as stated above, accurate results cannot be obtained by comparing the test on such a blood filtrate with the weaker standard diluted to 12.5 cc. With a little practise on the procedure outlined, it is possible to match the color of the test on unknown blood filtrates with that of the standard dextrose solution diluted to 25 cc. very rapidly, and in most instances in no more than two steps.

Since, in the procedure suggested, the total dilution of the test on unknown blood filtrates is not carried out all at once, but by the addition of smaller amounts of water several times, it is necessary in mixing the contents to invert the tube more often than in the original method. It seemed possible that, under these circumstances, enough fluid would be lost on the stopper and around the mouth of the tube to introduce an appreciable error. This, however, was not found to be so. A tube filled with water inverted fifteen times, the stopper being removed after each third inversion, showed no diminution in the volume of its contents that could be made out on gross inspection. This number of inversions is sufficient to mix thoroughly with its contents water added to a tube five different times, more than is ever necessary in carrying out the procedure presented above.

#### SUMMARY.

If correct values are to be obtained with the Folin and Wu blood sugar determination it is essential, for the colorimetric comparison, to have the color finally developed by the blood filtrate being tested very nearly the same as that developed by the standard dextrose solution with which it is to be compared. This necessitates for accurate results the use of more standard solutions than is convenient. In this paper the details of a technique are presented in which the color developed by the unknown blood filtrate with the Folin and Wu method is the same as that of the standard dextrose solution when the colorimetric readings are made; and one standard may be used for many bloods, and two or at most three make possible accurate determinations of the dextrose in any blood every encountered. The modification here presented consists simply in diluting the unknown in the test-tube after the addition of the phosphomolybdate-phosphotungstate sugar reagent and development of color until it is approximately the same color as the standard, and including this variable factor in the formula for calculating the final results.



## INSULIN AND PHLORHIZIN DIABETES.

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The advent of *insulin* and its now well recognized effects upon diabetes of the pancreatic type, afford a new test whereby the characteristics of phlorhizin diabetes and pancreatic diabetes may be compared. All earlier studies of the diabetic state induced by phlorhizin have confirmed its unique character; and recently Nash and Benedict (1) advanced the view that phlorhizin effects an intrinsic impairment of the sugar-burning mechanism. If this hypothesis is true, insulin—so far as its properties are now known—should not cause the utilization of sugar by the phlorhizinized animal; or, at most, should produce a very limited effect (such as might be attributed to a transient mass action factor). Accordingly, the administration of insulin to phlorhizinized dogs was undertaken by the present writer. While several details of the problem are still being investigated it has seemed desirable to submit now the results so far obtained.

### GENERAL PROCEDURE.

Fasting female dogs were employed in the experiments. Beginning on the 3rd day of fasting the animals were injected subcutaneously with an olive oil suspension of phlorhizin, at intervals as shown in the tables. On the 3rd phlorhizin day an injection of insulin was given.

All urines were taken by catheter (unless otherwise noted) and were analyzed in duplicate. Total nitrogen was determined by the macro Kjeldahl procedure, and total reducing sugar by the Allihn method.

Blood was obtained through an aspirating needle from the jugular vein. Potassium oxalate was employed as the anticoagulant.



TABLE I.  
Dog 1. Female Weight 11.5 kilos. Last feeding, 9.00 a.m., Mar. 22. Beginning Mar. 24, 1.0 gm. of phlorhizin in olive oil was injected subcutaneously at 9.00 a.m. daily

Date.	Time of collection.	Urine.						Jugular vein blood sugar per 100 cc		Remarks
		Vol-ume.	Nitrogen		Sugar		D N	Bene-dict method	Folin-Wu method	
			Total	Per hr	Total	Per hr				
		cc	gm	gm	gm	gm		mg	mg.	
Mar. 23	9 00 a m 10 00 " 11 00 " 3 00 p m							124 80 70 97	81 52 43 70	0 5 cc. of H-10 Iletin (5 units) injected immediately after drawing blood at 9 00 a m. Symptoms of weakness quickly manifested, persisting for about 5 hrs.
Mar. 24	9 00 a m to							124	83	
Mar. 25	9 00 a m	445	8 25	0 34	27 90	1 16	3 38			Urine lost.
Mar. 25	9 25 a m 12 00 n.	72	1 10	0 43	3 81	1 47	3 46			
Mar. 26	6 10 p m	106	2 55	0 41	8 05	1 30	3 16			
	7 00 a.m.	205	5 46	0 42	16 51	1 28	3 02			
	9 00 "	35	0 84	0 42	2 57	1 28	3 07	95	60	
Total .....		418	9 95	0 42	30 94	1 31	3 11			
Mar. 26	11 00 a.m. 1 00 p.m. 6 00 "	150 66 122	1 03 0 85 1 77	0 51 0 43 0 35	3 16 2 56 4 23	1 58 1 28 0 85	3 07 3 01 2 39	70	40	0 5 cc of H-10 Iletin injected at 11.00 a.m. Animal showed no apparent symptoms until about 2 00 p m, when it vomited and suddenly seemed very ill and weak.
Mar. 27	9 00 a.m. to	216 85*	2 77 2 17	0 33	11 76 7 19	1 26 3 31	4 26 3 31	96	56	Condition slightly improved at 6.00 p.m.; unusually good next morning.
Total.....	.....	639	8 59	0 36	28 90	1 20	3 36			

\* The two urines, obtained between 6.00 p.m., Mar. 26, and 9.00 a.m., Mar. 27, were for indeterminate separate intervals, the first being collected in the cage during the night and the second by catheter.

Each blood specimen was analyzed in duplicate for sugar, both by the Benedict (2) and the Folin-Wu (3) methods.

Merck's phlorhizin and the "H-10 Iletin," as supplied by Eli Lilly and Company, were used. The potency of the Iletin was ascertained in each case by a preliminary injection on the 2nd fasting day.

#### EXPERIMENTAL.<sup>1</sup>

Protocols of the experiments are given in Tables I and II. The experimental plan was the same for the two dogs, and the results in the two cases are essentially identical. Since, however, the results for Dog 3 are somewhat more complete than for Dog 1 discussion will be confined chiefly to the former.

In presenting the blood sugar findings only the values given by the Benedict method will be dealt with. It is of interest to note, however, the approximately constant difference in the two sets of values at different levels.

Dog 3 was a vigorous, healthy female which had been kept in the laboratory for several weeks at practically constant weight on a diet of dog cakes. After a feeding at 9.00 a.m., May 17, the animal received no food until the termination of the experiment, 6 days later.

At 11.03 a.m., May 18, the animal received subcutaneously in the abdominal region 0.75 cc. of "H-10 Iletin," supposedly equivalent to 7.5 units of insulin. The dosage was measured as accurately as possible in a tuberculin syringe. A control blood, 30 minutes previously, contained 0.122 per cent of sugar. 2 hours after the Iletin injection the blood sugar had fallen to 0.083 per cent, but after a further interval of 4 hours was again at the initial level, 0.122 per cent. (In the case of Dog 1 recovery of the initial blood sugar level was delayed beyond 6 hours.) Coincidentally with the fluctuations in the blood sugar concentration there was an unmistakable, inverse variation in the rate of nitrogen excretion in the urine. In two 2 hour control periods preceding the Iletin dosage the average hourly excretion of nitrogen was 241 and 234 mg., respectively. For the first subsequent 2 hour period this rate was practically unchanged (241 mg.); but in the next follow-

<sup>1</sup> The writer is indebted to Mr. B. K. Harned, of this department, for technical assistance in the work.

TABLE II

Dog 3. Female. Weight 15.4 kilos. Last feeding, 9.00 a. m., May 17. Beginning May 19, 1.0 gm. of phlorhizin in olive oil was injected subcutaneously at 9.00 a. m. daily

Date	Time of collection	Urine						Jugular vein blood sugar per 100 cc		Remarks
		Vol- ume	Nitrogen		Sugar		D N	Bene- dict method	Folin- Wu method	
			Total	Per hr	Total	Per hr				
May 18	7.00 a.m. to 9.00 a m	cc	gm	gm	gm	gm	mg	mg		
		50	0.48	0.24						
May 18	10.30 a.m.							90	0.75 cc of H-10 Iletin (7.5 units) injected at 11.03 a.m. Marked symptoms of drowsiness and weakness by 12.00 n.; very pronounced by 1.00 p.m.; condition slightly improved by 3.00 p.m.; practically normal again by 5.00 p.m.	
	11.00 "	50	0.47	0.23			122			
	1.00 p.m	26	0.48	0.24			83	52		
	3.00 "	33	0.60	0.30						
May 19	5.00 "	47	0.58	0.29			122	91		
	7.00 "	36	0.45	0.22						
	9.00 a.m	150	2.51	0.18						
		342	5.09	0.21						
Total . . .										
May 19	1.00 p.m	120	0.99	0.25	5.92	1.48	5.98			
May 20	7.00 "	220	1.89	0.31	9.30	1.55	4.92			
	9.00 a.m.	490	5.56	0.40	19.08	1.36	3.43			
Total.....		830	8.44	0.35	34.30	1.43	4.06		Combined cage and catheter urine.	

May 20	1.00 p.m.	164	1 67	0 42	6 42	1 60	3 84				
	7 00 "	315	2 88	0 48	10 03	1 67	3 48				
May 21	7.00 a.m.	355	5 21	0 43	18 22	1 52	3 49				
	9 00 "	70	0 84	0 42	3 34	1 67	3 97				
Total . . . . .		904	10 60	0 44	38 01	1 53	3 58				
May 21	10 00 a.m.							96	75		
	11 00 "	80	0 88	0 44	3 64	1 82	4 14				
	1 00 p.m.	64	0 77	0 38	3 13	1 56	4 06	80	48		
	3 00 "	34	0 68	0 34	1 82	0 91	2 68				
	5 00 "	154	0 77	0 38	1 90	0 95	2 47	71	47		
	7 00 "	84	0 68	0 34	3 05	1 52	4 49				
	9 00 "	68	0 54	0 27	3 66	1 83	6 77				
May 22	7 00 a.m.	260	2 90	0 29	14 96	1 50	5 16				
	9 00 "	75	0 79	0 39	2 68	1 34	3 39	91	62		
Total.....	. . . . .	819	8 01	0 33	34 84	1 45	4 35				
May 22	1 00 p.m.	165	1 83	0 46	6 17	1 54	3 36				
	7.00 "	230	2 81	0 47	8 62	1 44	3 07				
May 23	7.00 a.m.	320	5 07	0 42	16 39	1 37	3 23				
	9 00 "	52	0 84	0 42	2 50	1 25	2 98				
Total. . . . .	....	767	10 55	0 44	33 68	1 40	3 19				

1.0 cc of H-10 Iletin (10 units) injected at 11 00 a.m. Maximal symptoms observed from about 12 00 n. to 2.30 p.m., but dog remained drowsy and weak for about 10 hrs.

Dog in splendid condition at end of experiment.

ing 2 hour period the rate suddenly jumped to 300 mg. per hour, an increase of about 25 per cent. From this maximum there was a gradual drop to an average of 179 mg. per hour for the 14 hours which close this day. Probably the data do not show the lowest level of nitrogen excretion attained, nor is it possible to say whether the drop was in any degree compensatory or directly influenced by the iletin injection. Normally, this would be a period of diminishing nitrogen excretion by a dog previously well nourished. We incline to the view that the temporary rise noted in the nitrogen excretion reflects an increased nitrogen catabolism stimulated by the fall below the existing level of the blood sugar. The phenomenon would thus be comparable to the increased protein metabolism in phlorhizin diabetes, where there is a continuing hypoglycemia. Ringer (4) has shown that in the latter condition ". . . the diminution in the concentration of glucose in the blood is an essential factor in the rise of protein metabolism."

The physical symptoms parallel the blood sugar variations. Within about 1 hour after the iletin injection symptoms of weakness and drowsiness were apparent. The dog curled up in the cage and refused to stir or rise at call. By 1.00 p.m. these conditions were very pronounced. The respiration was very slow and deep. A little later muscular twitchings and labored heart action were observed. At 3.00 p.m. the state of the animal was apparently somewhat improved, and 2 hours later was again apparently normal.

Beginning May 19, the animal received daily at 9.00 a.m. subcutaneous injections of 1 gm. of phlorhizin in olive oil. In the first two subsequent 24 hour periods we have the usual picture of rising nitrogen metabolism and falling D:N quotients.

During the second 24 hour phlorhizin period the D:N quotients were reasonably constant, the average ratio for the period being 3.58. It is true that in the two control urines from 7.00 to 11.00 a.m., May 21, the D:N values were somewhat elevated—3.97 and 4.14, respectively; but in our experience such rises in the morning ratios are the rule rather than exception, and are frequently observed even after more prolonged phlorhizin administration.

The blood sugar at 10.00 a.m., May 21, was 0.096 per cent, as compared with the fasting prephlorhizin value of 0.122 per cent. 1 hour later the animal received subcutaneously, in the abdomen,

1.0 cc. of "H-10 Iletin" (10 units). At 1.00 p.m. the blood sugar was 0.080 per cent; at 5.00 p.m., 0.071 per cent; and at 9.00 a.m., May 22, 0.091 per cent.

The physical symptoms following iletin injection were quite similar to, but more severe than, those noted in the prephlorhizin test. These symptoms of weakness and drowsiness were observable within 30 minutes of the injection and were at a maximum from about 12.00 n. to 1.00 p.m. During this hour the animal was practically comatose, and doubt was felt that it would survive. Later in the afternoon there was rapid recovery; at 5.00 p.m. the animal seemed definitely over the iletin effect. Still later there was a moderate relapse, but the following morning the animal was found in very good condition.

Following the injection of iletin there occurred a marked disturbance of the D:N ratios. With the falling blood sugar there was a sharply diminished excretion of sugar in the urine. The hourly rate decreased from 1.82 gm. for the 2 hour control period ending at 11.00 a.m., to about 0.90 gm. for the period from 1.00 to 5.00 p.m. Then occurs a sharp rebound which extends temporarily above the average control rate. Coincidentally with the fall in sugar elimination, the rate of nitrogen excretion also drops off. While this drop is not so rapid it is much more prolonged, reaching the lowest level in the urine from 7.00 to 9.00 p.m. Here the hourly rate of nitrogen elimination is 272 mg. as compared with the control rate of 440 mg. Thus the D:N quotients first fall, then rise, and finally fall again to the uncomplicated phlorhizin level. The lowest D:N ratio, 2.47, occurs in the urine from 3.00 to 5.00 p.m., and the highest, 6.77, in the 7.00 to 9.00 p.m. urine.

#### DISCUSSION.

In our opinion the most striking phenomenon in these experiments is the marked diminution in nitrogen excretion by the phlorhizinized dog following injection of insulin. (We may contrast this phenomenon with the increased nitrogen metabolism following insulin administration to the fasting non-phlorhizinized dog.). In Dog 3 the total urinary nitrogen was, for the 24 hours immediately preceding the insulin period, 10.60 gm.; for the 24 hour period in which insulin was given, 8.01 gm.; and for the immediately succeeding 24 hour period, 10.55 gm. In other words,

following the administration of 10 units of insulin there was a decrease of 2.5 gm. in the total nitrogen of the urine. But two possible explanations of this effect suggest themselves to us: Either insulin induces a specific retention of nitrogen (as by lessening kidney permeability); or there is a sparing action upon the protein metabolism. The first of these possibilities is unlikely, since there is little indication of a compensatory rise in nitrogen excretion during the postinsulin period. Thus the average hourly excretion of nitrogen was, for the 24 hour fore period, 441 mg.; for the 24 hour insulin period, 334 mg.; and for the 24 hour after period, 439 mg.

On the other hand, if the protein metabolism is spared, we might expect just the order of disturbance in the D:N ratios that we do in fact find. Nash and Benedict (1) have shown that the phlorhizinized dog eliminates sugar more rapidly than urea which originates simultaneously. Consequently, any changes in the rate of nitrogen metabolism are more quickly reflected in the urinary sugar than in the urinary nitrogen. Changes in the urinary nitrogen are appreciably retarded by reason of the "nitrogen lag." Hence, with the D:N at a certain value, if the protein metabolism is suddenly diminished the D:N will first fall below the initial value, then rise above the initial value, and finally be reestablished at the initial value. Furthermore, if the protein metabolism is first depressed and then rises again before the above series of readjustments in the D:N quotients is completed, the elevation of the intermediate quotients may be exaggerated, and the reestablishment of the normal quotient may be considerably postponed. It is apparently just such a series of fluctuations that we find in the case of Dog 3 following the insulin dosage. When the lowest level of nitrogen excretion was found in the urine from 7.00 to 9.00 p.m., May 21, the rate of nitrogen metabolism was probably already recovering from its temporary depression; but the final elimination of the increased nitrogenous waste was seemingly carried over into the subsequent 24 hour period.

But if the second possibility is true, how may the sparing of the protein metabolism be explained? Ringer (4) has analyzed protein metabolism into three components: (1) The "wear and tear" quota; (2) the "dextrose nitrogen" which represents the amount of protein catabolized in excess of the "wear and tear"

quota when the calorific requirement is covered exclusively by fat; and (3) the "complementary nitrogen" which represents a further increment of protein catabolized for dynamogenetic purposes in fasting. The nitrogen metabolism in phlorhizin diabetes is a function of all these components.

The "complementary nitrogen" may be spared by any foodstuff; we may consider first the hypothesis that insulin made possible the combustion of some carbohydrate. Our data do not positively deny or confirm this possibility; it would seem that only respiratory and calorimetric measurements could definitely decide the question. But, certainly, if carbohydrate was burned the amount was very small, and this despite the prolonged effect upon the blood sugar concentration of an amount of insulin calculated to make available to the diabetic of the pancreatic type up to 40 gm. of carbohydrate. The total sugar output in the urine for the 24 hour insulin period was 34.84 gm. as compared with 38.01 and 33.68 gm. in the fore and after periods, respectively. Furthermore, the average D:N of the 24 hour insulin period was 4.35, a significantly high value. Indisputably, sugar was withdrawn from the blood under the influence of insulin, and, consequently, we find for this part of the period a sharply diminished elimination of sugar in the urine; but that the sugar so withdrawn was later surrendered again to the blood is indicated by the sudden rise in sugar excretion beginning about 6 hours after the injection of insulin. We believe these findings substantially corroborate the view that phlorhizin effects an intrinsic impairment of utilization of sugar by the tissues.

If carbohydrate is not burned but is only temporarily withdrawn from the blood and concentrated in the tissues, whether as glucose or glycogen or some other form, would this circumstance account for a sparing effect upon the protein metabolism? It is difficult to see how the "complementary nitrogen" component could thus be affected, since this involves dynamogenetic equivalents. But it would seem that the "dextrose nitrogen" component might be diminished. It has been shown repeatedly that increasing the concentration of sugar in the tissues of the phlorhizinized dog, by feeding glucose, spares the protein metabolism though none of the ingested sugar is burned; if, under the action of insulin, carbohy-



drate is concentrated in the tissues at the expense of the blood sugar, we should expect a similar sparing influence.

There remains the possibility that the "complementary nitrogen" component may be spared at the expense of an increased or more complete fat metabolism. One of the most remarkable effects of insulin in pancreatic diabetes is the very prompt disappearance of acetone bodies from the urine, and the increase in the carbon dioxide-combining power of the blood. These changes may—in fact usually do—occur before the urinary sugar is brought down to a normal level. In both Dogs 1 and 3 it was observed that the urines became distinctly less acid to litmus following insulin injections; but at the time this fact did not impress us strongly. The urine of Dog 3 from 1.00 to 3.00 p.m., May 21, was neutral to litmus; in the immediately preceding and following 2 hour urines the reaction was faintly acid; and at all other times the urine reaction was strongly acid. Not having anticipated the possibility of a direct effect of insulin upon the fat metabolism we were not prepared at the time to follow out the above observations. However, we are now engaged in investigating further this and other questions raised herein.

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# THE ACTION OF AMMONIUM HYDROXIDE AND OTHER ALKALINE COMPOUNDS UPON INSULIN.\*

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This paper is a report upon our observations on the lability<sup>1</sup> of insulin in the presence of alkali. These experiments were undertaken, first, in order to determine the influence of reaction conditions upon the activity of insulin, as a rational preparation for later attempts to isolate a definite chemical compound having insulin properties; and second, in order to obtain some hint, if possible, as to the chemical nature of the group or groups which contribute the particular property which characterizes insulin.

*1. Action of Ammonium and Potassium Hydroxides on Insulin.*—In Tables I and II are given the results of the action of ammonium hydroxide and potassium hydroxide upon insulin, which we prepared from fresh beef pancreas by the Collip method. The preparations were injected into rabbits that had been fasted during the 18 hours previous to injection. The blood sugar concentrations were determined before and again 2 hours after injection, unless the rabbits suffered convulsions, in which case

\* We wish to express our gratitude to various executives in Swift and Co., Chicago, for supplying us, without cost, with the beef pancreas used in this and other work upon insulin done in this laboratory.

<sup>1</sup> Best and Macleod (Best, C. H., and Macleod, J. J. R., *J. Biol. Chem.* 1923, lv, p. xxix) say that: "Moderate degrees of alkalinity (to litmus) do not affect the strength of insulin during a period of 6 minutes at room temperature. We cannot as yet state the degree of alkalinity which insulin can withstand." This is the only statement that we have found in the literature on the behavior of insulin with alkali (Since this paper was sent in for publication Dudley (Dudley, H. W., *Biochem. J.* 1923, xvii, 376) has published some data on the destruction of insulin by NaOH solutions and by boiling Na<sub>2</sub>CO<sub>3</sub> solutions.)

TABLE I.  
*Experiments with Ammonium Hydroxide.*

Injected 1 cc. into  $\pm 2$  kilo fasted rabbits.

No.	Material	Date	Blood sugar		Remarks.
			Before.	After.	
			per cent	per cent	
1	1.5 cc. insulin No. 45 + 2.5 cc 1.0 N $\text{NH}_4\text{OH}$ + 1.0 cc. $\text{H}_2\text{O}$ . (Mixture 0.50 N $\text{NH}_4\text{OH}$ .) Started Mar. 28.	Mar. 29	0 20	0 08	After 2 hrs.
		Apr. 3	0 14	0 14	" 2 " Acidified the solution with HCl Apr. 4.
		" 5	0 11	0 02	After 1 hr. (in convulsions).
		" 7	0 12	0 05	" 2 hrs.
2	Started above experiment again on Apr. 5.	" 12	0 12	0 07	" 2 " Acidified with HCl Apr. 12.
		" 13	0 11	-0 02	" 1½ " (in convulsions).
		" 9	0 14	0 08	" 2 "
3	Same experiment started again Apr. 7	" 12	0 12	0 06	" 2 " Acidified with HCl Apr. 12.
		" 13	0 13	-0 02	" 45 min. (in convulsions).
		" 9	0 14	0 08	" 2 "
4	1.5 cc. insulin No. 52 + 3.5 cc. 1.0 N $\text{NH}_4\text{OH}$ . Started May 14.	May 16	0 12	0 13	" 2 hrs.
		" 17	0 14	0 12	" 2 " Acidified with HCl and injected 1 hr. later May 18.
		" 18	0 12	0 08	After 2 hrs.
		" 19	0 10	0 08	" 2 "
		" 21	0 10	0 05	" 2 "

5	Started again with No. 52 May 19.	May 21 " 22	0 10 0 11	0 03 0 03	After 2 hrs. " 2 " (in convulsions)
6	1.5 cc. insulin No. 47 + 3.5 cc. 1.0 N NH <sub>4</sub> OH. Started May 19.	" 21 " 22 " 23	0 11 0 16 0 12	0 06 0 12 0 08	" 2 " " 2 " " 2 " Acidified with HCl and injected at once May 23.
		" 23	0 12	-0 02	After 2 hrs. (in severe convulsions).

blood was taken when convulsions were first observed as stated in the tables under the heading, "Remarks." On the dates indicated the alkaline solutions were acidified with hydrochloric acid (*i.e.* made distinctly acid to litmus) and subsequently injected again to determine if the insulin activity had been restored. That 0.3 cc. of the preparations used in these experiments always produced pronounced convulsions was determined by control experiments with the preparations in which 1.5 cc. were diluted with 3.5 cc. of water so that 1 cc. of dilute solution could be injected as in the alkali experiments. These control results are not given.

In Experiment 1 the insulin had become completely inactive in 6 days. When it was acidified, on the supposition that a reversible reaction is involved rather than a destructive reaction such as saponification or hydrolysis, the animal was thrown into convulsions a little sooner than it normally was by the unchanged original preparation. The blood sugar was also reduced to the level characteristic of convulsions. This experiment was the first one made and it was some time before we obtained the same result again.

In Experiment 2 the insulin activity was reduced considerably in a week. Since about 0.2 cc. of the original preparation lowered blood sugar to 0.03 per cent and produced convulsions, it was about 50 per cent inactivated in this experiment. When acidified it recovered its original strength fully. The same result was obtained in Experiment 3.

With insulin preparation No. 52 a little more ammonium hydroxide was added and the insulin became completely inactive in 2 days. This was confirmed on another rabbit the next day. The solution was then acidified and injected an hour later. Its activity was partially restored. It remained unchanged when injected the next day, but 2 days later had apparently regained somewhat more of its original activity.

In Experiment 5 we attempted to repeat No. 4, but the insulin lost nothing in activity in 3 days although the materials used were all portions of those that had been used in Experiment 4. Out of 30 or more experiments we obtained this result but once. It may have been due to the loss of ammonia around a loose stopper.

TABLE II.  
*Experiments with Potassium and Sodium Hydroxides.*  
 Injected 1 cc. into  $\pm 2$  kilo rabbits fasted 18 hours.

No.	Material	Date	Blood sugar		Remarks.
			Before	After	
			per cent	per cent	
7	1.5 cc. insulin No. 45 + 2.5 cc. 1.0 N KOH + 1.0 cc. H <sub>2</sub> O. (Mixture 0.50 N KOH.) Started Mar. 28.	Mar 29 Apr. 5	0 15 0 13	0 12 0 13	After 2 hrs. Acidified with HCl Apr. 4. " 2 "
8	Started Experiment 7 again Apr 7.	" 9 " 11	0 13 0 13	0 12 0 13	" 2 " Acidified with HCl Apr. 10. " 2 "
9	1.5 cc. insulin No. 45 + 2.5 cc. 0.1 N KOH + 1.0 cc. H <sub>2</sub> O (Mixture 0.05 N KOH) Started Apr 14.	" 16 " 19 " 20	0 13 0 11 0 10	0 05 0 06 0 06	" 2 " Acidified with HCl Apr. 19. " 2 " " 2 "
10	Experiment 9 with 0.1 N NaOH Started Apr. 14.	" 16 " 19	0 11 0 14	0 05 0 05	" 2 " " 2 "
11	Repeated Experiment 10 with insulin No. 52. Started May 14.	May 16 " 17 " 19	0 11 0 14 0 10	0 06 0 12 0 10	" 2 " Acidified with HCl May 18. " 2 " " 2 "

In Experiment 6 a much older preparation was practically entirely inactivated and restored to full strength when injected at once after acidifying. The last two injections in this experiment were made within a few minutes of each other.

The results of the experiments with potassium hydroxide require little comment. In Experiments 7 and 8 inactivation was complete and not reversible upon acidification. In Experiments 9 and 10, which contained one-tenth as much alkali, inactivation was slower but, as found in Nos. 9 and 11, irreversible upon acidification even after standing 2 days. In Experiment 11 inactivation was complete after 3 days, although in Experiment 10 inactivation was not well under way even in 5 days.

*2. Action of Disodium Phosphate, Sodium Carbonate, and Bicarbonate on Insulin.*—Some experiments were made on the action of these compounds upon insulin. The experiments were not extended since no pronounced effects were observed. In general, the prolonged action of these substances in the concentration used diminished the activity of the insulin enough to do away with the symptoms of convulsions although the blood sugar was reduced to about the level for convulsions. When acidified, however, these solutions that had been somewhat "dampened" by the alkali compounds were restored to full activity. In fact, they often seemed to be stronger in their effects than the controls with the untreated insulin.

*3. The Velocity of Inactivation and Reactivation of Insulin at Room Temperature.*—In order to determine the velocity of reactivation of insulin completely inactivated by ammonium hydroxide, the preparation in Experiment 6 was acidified and injected as quickly as possible afterward (*i.e.* within a minute or so). The results showed that insulin was restored to full activity by the time it had been absorbed from the point of injection. In the rabbit that received the inactive preparation there was a slight reduction of blood sugar, indicating that perhaps a little unchanged insulin remained in solution. In the other rabbit, this preparation, after having been acidified, behaved as though it had never been inactivated. A similar and more perfect result of this kind is recorded in Experiment 19. The results on the action of ammonium hydroxide show that insulin requires at least several days to be completely inactivated

TABLE III  
*Experiments with Sodium Phosphate, Carbonate, and Bicarbonate.*  
 Injected 1 cc. into  $\pm 2$  kilo rabbits fasted 18 hours.

No.	Material	Date	Blood sugar		Remarks.
			Before	After	
			per cent	per cent	
12	1.5 cc. insulin No. 45 + 3.5 cc. m/3 $\text{Na}_2\text{HPO}_4$ . Started Mar. 28.	Mar. 29 Apr. 3	0 11 0 15	0 02 0 09	After 1½ hrs. (in convulsions). " 2 "
13	Repeated Experiment 12. Started Apr. 7.	" 12 " 13	0 14 0 14	0 07 -0 02	" 2 " Acidified with HCl Apr. 12. " 1 hr. (in convulsions).
14	1.0 cc. insulin No. 52 + 2.5 cc 5 per cent $\text{Na}_2\text{CO}_3$ solution. Started May 10.	May 11 " 15	0 11 0 09	0 04 0 03	" 1½ hrs. ( " ) " 1½ " ( " )
15	1.0 cc. insulin No. 52 + 1.0 cc 5 per cent $\text{Na}_2\text{CO}_3$ solution. Started May 10. (Injected 0.6 cc)	" 11 " 15	0 11 0 10	0 07 0 03	" 2 " " 1½ " (in convulsions).
16	1.5 cc. insulin No. 45 + 3.5 cc. m/3 $\text{NaHCO}_3$ solution. Started Apr 7	Apr. 12 " 13	0 13 0 11	0 03 -0 02	" 2 " Acidified with HCl Apr. 12. " 1½ " (in convulsions).



in our experiments, but does not require more than an hour or so, at most, to be completely reactivated.

4. *Attempts to Catalyze the Action of Alkali on Insulin.*—In Section 6 the action of ammonium hydroxide upon insulin is discussed as a keto-enol rearrangement. As is well known, such rearrangements are subject to catalytic influence in both directions. We were therefore interested in determining to what extent the inactivation of insulin by alkalis is subject to catalysis.

In a study of the action of alkali upon the oxidation of butyric acid<sup>2</sup> it was found that although potassium hydroxide alone is incapable of catalyzing the oxidation of butyric acid it promotes the action of ammonium hydroxide and may be substituted for part of it. Our first experiments were therefore made with mixtures of ammonium and potassium hydroxides.

The result in Experiment 18, especially, was surprising. The quantity of potassium hydroxide added (0.0028 gm.) was only one-fifth of that added in Experiment 10 in which insulin was not much inactivated even after some days. The effect of the addition of these small amounts of potassium hydroxide was to catalyze the permanent inactivation of insulin by ammonium hydroxide and to convert the reversible ammonium hydroxide effect into the irreversible potassium hydroxide effect. The result was a catalysis of the destructive reaction of alkali by ammonium hydroxide.

When Experiment 18 was repeated it was confirmed several times and then we finally obtained the results given in Experiment 19. This result is nearly 100 per cent different from that of the preceding experiment, and constitutes a catalysis of the reversible ammonium hydroxide effect.

Similar experiments were made using only 0.01 cc. of 1.0 N KOH, but the results so far obtained show no distinctive difference in comparison with the results obtained with ammonium hydroxide alone.

Other experiments were made substituting 0.2 to 1.75 cc. of the 1.0 N  $\text{NH}_4\text{OH}$  with 5 per cent  $\text{Na}_2\text{CO}_3$ . As near as we can tell the most frequent effect of the  $\text{Na}_2\text{CO}_3$  is to prevent the inactivation of insulin by ammonium hydroxide.

<sup>2</sup> Witzemann, E. J., *J. Biol. Chem.*, 1921, xlix, 123.

TABLE IV  
*Experiments with Mixed Alkalies.*

Injected 1 cc. into  $\pm 2$  kilo rabbits fasted 18 hours.

No.	Material	Date	Blood sugar		Remarks
			Before	After	
			per cent	per cent	
17	1.5 cc. insulin No. 47 + 3.32 cc. 1.0 N $\text{NH}_4\text{OH}$ + 9.18 cc 1.0 N KOH. Started May 24.	May 25 " 26 " 28	0 12 0 14 0 14	0 13 0 13 0 14	After 2 hrs " 2 " " 2 " Acidified May 25.
18	1.5 cc insulin No. 47 + 3.45 cc 1.0 N $\text{NH}_4\text{OH}$ + 0.05 cc 1.0 N KOH. Started June 6.	June 7 " 8	0 13 0 12	0 14 0 12	" 2 " " 2 " Acidified June 7.
19	Repeated Experiment 18 with insulin No. 49. Started June 27	" 28 " 29	0 13 0 13	0 12 0 04	" 2 " " 2 " Acidified just before injection. (no convulsions).

In brief, these preliminary results indicate that the effects of mixed alkalis do give rise to catalytic effects subject to the same unexplained vagaries as the simple alkalis.

5. *The Nature of the Chemical Action of Alkaline Compounds on Insulin.*—The results in the preceding sections show two distinct types of effects of alkaline compounds upon insulin, and possibly a third.

a. One of these is the striking reversible inactivation of insulin by ammonium hydroxide, which will be briefly discussed in the next section.

b. Another reaction which is typically obtained with potassium and sodium hydroxides is an irreversible destructive reaction. The writers are under the impression that this reaction is possibly an autohydrolysis due to the alkali, analogous to the autohydrolysis of alkali caseinates studied by Walters,<sup>3</sup> or some other reaction not reversible by acid.

c. The peculiar effect of sodium carbonates and phosphate was that they did not often (in the 25 to 30 observations made) appear to modify greatly the capacity of insulin preparations to lower the blood sugar, but did generally diminish what may perhaps be considered to be secondary effects; namely, convulsions. On the contrary, when the same preparations were acidified they appeared to cause somewhat greater lowerings in the blood sugar and more profound convulsions than the same preparation gave in the control experiments. Acidification of the untreated insulin preparation used in the control experiments did not give any pronounced increase in the effect. We are disposed to consider that in these alkaline preparations the chemical group in the insulin molecule that gives rise to these secondary effects becomes masked by condensing with other molecules present in the preparation, but that this does not effectively interfere with the action of the same or another group upon the sugar of the blood.

Our experiments with ammonium hydroxide as a whole gave three types of results: (1) the insulin was not appreciably inactivated; (2) the insulin was reversibly inactivated; and (3) the insulin was irreversibly inactivated. The first type of results

<sup>3</sup> Walters, E. H., *J. Biol. Chem.*, 1912, xii, 43; cf. also Mann, G., *Chemistry of the proteids*, London, 1906, 90, 337.

was obtained occasionally and is mentioned here for completeness because we are not yet sure that we can always avoid it and get the second type of effect. More than half the time we got the mixed effect of Types 2 and 3. Our difficulty was to get to Type 2 without going on to Type 3. We were, therefore, unable to predict in any given case which result would be obtained, even when the same materials were used. Owing to the impossibility of controlling all the variables that seem to be involved (such as the purity of the insulin, especially) we have contented ourselves with a qualitative survey of this aspect of the problem for the present, and hope to get a clearer insight into the factors involved by widening rather than intensifying our attack. Enough of our characteristic results have been included in this paper to illustrate all the effects observed.

6. *Possible Interpretation of the Action of Ammonium Hydroxide upon Insulin.*—The available chemical results upon insulin are as yet far too meager to provide a satisfactory basis for the interpretation of the reversible transformation of insulin described above. It should, however, not be misleading to consider briefly what the results obtained suggest concerning the constitution of insulin.

The definite chemical facts developed in this paper clearly suggest that the reversible effect of ammonium hydroxide involves a tautomeric rearrangement such as  $\cdot\text{CO}\cdot\text{NH}\cdot\rightleftharpoons\text{C}(\text{OH})\cdot\text{N}\cdot$  or  $\cdot\text{CO}\cdot\text{CHR}(\text{or H})\rightleftharpoons\text{C}(\text{OH})\cdot\text{CR}(\text{or H})$ . These two groups are mentioned solely because the first is the most abundantly well known tautomeric group occurring in protein-like compounds, such as insulin appears to be,<sup>4</sup> and the second is the hitherto most abundantly studied rearrangement of this kind.<sup>5</sup>

When the results are considered in this way, interesting and important analogies between insulin and other biochemical catalysts are suggested. Thus the pioneering results of von Euler and Svanberg<sup>6</sup> on the reactivation of "poisoned" or inactivated

<sup>4</sup> Witzemann, E. J., and Livshis, L, *J. Biol. Chem.*, 1923, lvii, 425.

<sup>5</sup> Meyer, V., and Jacobson, P, *Lehrbuch der organischen Chemie*, Leipsic, 2nd edition, 1902, iii, 1137. Meyer, K. H., *Ann. Chem.*, 1911, cccclxxx, 212; Meyer, K. H., and Kappelmeier, P., *Ber. chem. Ges.*, 1911, xlv, 2718; 1912, xlv, 2843. Knorr, L, Rothe, O., and Averbach, H., *Ber. chem. Ges.*, 1911, xlv, 1138. Dimroth, O, *Ann. Chem.*, 1904, cccxxxv, 14.

<sup>6</sup> von Euler, H., and Svanberg, O., *Fermentforsch.*, 1919–20, iii, 330.

enzymes are of special interest in this connection. The poisoning effect of heavy metals ( $\text{HgCl}_2$  and  $\text{AgNO}_3$ ) upon invertase is completely reversible. Their results on the reversible inactivation of invertase by eleven amines<sup>7</sup> led them to consider that the aldehyde group determines this property of invertase. Meanwhile, Wöker and Maggi<sup>8</sup> consider for entirely different reasons that the effects of peroxidase, catalase, reductase, and diastase are definitely due to the aldehyde group and have proposed formaldehyde as a chemical model for these four types of enzymes, since it shows all these types of chemical activity.

The results cited above as well as other work that is not mentioned are interesting in connection with insulin mainly because they suggest ways of extending the study of the labile group involved in the reversible transformation described.

#### SUMMARY.

The results described in this paper show that insulin is more or less completely inactivated at room temperature by 0.5 or 0.7 N ammonium hydroxide in the course of some days and that its original activity is usually quickly restored on acidifying the ammoniacal solution with hydrochloric acid. It was also found that sodium and potassium hydroxides even at 0.1 N concentration inactivate insulin irreversibly at room temperature. Sodium carbonate and bicarbonate and disodium phosphate had almost no action upon insulin. Some exploratory experiments on the effects of mixed alkalis upon insulin indicate that the above reactions are also subject to secondary accelerative and retarding effects depending on the mixture used.

The known facts concerning the reversible inactivation of insulin by ammonium hydroxide suggest a tautomeric rearrangement. Nothing definite can yet be said as to the nature of the groups that would be involved in this rearrangement.

<sup>7</sup> von Euler, H., and Svanberg, O., *Formentforsch.*, 1920-21, iv, 29, 54, 143.

<sup>8</sup> Woker, G., and Maggi, H., *Ber. chem. Ges.*, 1919, lii, 1594. Maggi, H., *Formentforsch.*, 1919, ii, 304.

## ANIMAL CALORIMETRY.

### TWENTY-THIRD PAPER.

#### THE INFLUENCE OF THE METABOLISM OF THE NUCLEIC ACIDS ON HEAT PRODUCTION.

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The influence of the metabolism of the major foodstuffs—carbohydrates, proteins, fats—on heat production has a very extensive literature. Very little, however, has been reported on this aspect of the metabolism of the nucleic acids. It is reasonable to inquire into this subject. The nucleic acids are undoubtedly vital constituents of cells, yet the significance of their metabolism, or indeed of their derivatives, is little understood. What is the rôle of the exogenous nucleic acids in the body? There is good evidence that they are not needed for the synthesis of the nuclear materials of the cells (1). From the caloric standpoint they very likely have some nutritional value, but this must be quite inconsiderable in comparison with the energy derived from the major foodstuffs.

Estimating from the amount of uric acid eliminated, a normal adult may in a day metabolize from 2 to 4 gm. of nucleic acid. On a diet very rich in cellular materials, such as liver or sweetbread, the amount of nucleic acid utilized may reach more than twice that amount. Under pathological conditions, as in leucemia and in the resorption of leucocytic exudates, it may rise considerably higher. Assuming a maximal metabolism of 10 gm. of nucleic acid a day, the calories to be derived from this source are probably not more than 25.

30 per cent of the thymus nucleic acid molecule by weight is phosphoric acid. This can give no heat. 40 per cent is hexose. This substance might, therefore, yield 16 calories. The data on the caloric possibilities

of the purines are imperfect, although the intermediary metabolism is well understood. Guanine, in the bomb calorimeter (2), has a value of 3.9 calories, and uric acid 2.8 calories to the gram, so that the transformation from the former to the latter yields 0.8 calorie (1 gm. of guanine = 1 gm. of uric acid). It must be remembered that this value includes the heat of oxidation of the amino group to nitric acid. Physiologically the amino group goes over to urea without giving heat, hence the nutritional caloric value of guanine would be much less than 0.8 calorie. The heat value of adenine is unknown. Since in comparison to guanine it offers 1 more hydrogen atom for oxidation, its heat value must be somewhat higher. It appears reasonable to assume, then, a caloric value for the metabolism of 1 gm. of purine in the body of not more than 0.8 calorie. Hence, from 10 gm. of nucleic acid, which contains 20 per cent by weight of purine, might be derived about 1.5 calories. The caloric contribution of the pyrimidines is even more problematical, since their fate is little understood. No data are available on the heat value of the pyrimidines in the bomb calorimeter, but since they are structurally less complex than the purines it may be assumed that their caloric value in the bomb is less than that of the latter—not more perhaps than 4 calories to the gram. There is some evidence that they are metabolized to urea (3). This would cut their caloric value to about 1.5 calories to the gram in the body. Since they constitute 16 per cent of the nucleic acid molecule, about 3 calories from this source are probable. The total thus far is less than 21 calories hence the 25 calories allowed above are reasonable.

Are the nucleic acids, then, merely accidents of the diet that give the body small benefit and only the labor of their destruction and their elimination? Do they perhaps, in their metabolism, stimulate the cells to greater heat production; that is, have they a specific dynamic effect? There is a constant and considerable endogenous metabolism of nucleic acid derivatives, increased by exercise and reduced by rest (4). Is this concerned with the production of heat?

A calorimetric investigation of the conduct of the nucleic acids might yield information on this point. Accordingly, the effect of the ingestion of animal and plant nucleic acid on the basal metabolism of the dog was undertaken. Since the metabolism of these substances is not very great, it may be argued *a priori* that its influence on the heat production must therefore be very small. This does not follow. Lusk (5) has shown that 10 gm. of the amino-acid glycocoll, containing only 21 nutritional calories, will raise the metabolism 17 calories above the basal. On the other hand, 75 gm. of fat, containing 694 calories,

will elevate metabolism only 29 calories above the basal. Hence, only a calorimetric experiment might determine the extent, if any, of the influence of the nucleic acids on heat production.

#### EXPERIMENTAL.

Two highly trained female dogs (Nos. 18 and 19) were used. These had been employed in this laboratory for calorimetric investigation for several years. They were kept on a standard maintenance diet which was given at 5 o'clock in the afternoon. 18 hours later, that is the next morning, they were put in the calorimeter after receiving the substance whose influence on the heat production was to be determined.

It was necessary to give the nucleic acid in a soup of Liebig's extract in order to make it palatable. Hence, the basal metabolism was established after giving the animals such a soup containing 0.2 gm. of Liebig's extract. Lusk (6) has shown that the above mentioned amount of this substance does not affect the heat production appreciably.

The yeast nucleic acid used was Merck's commercial preparation, analysis of which showed a composition of 17 per cent of nitrogen and 8.2 per cent of phosphorus. The biuret test showed that it was practically free of proteins. This was sufficiently pure for our purpose. 20 gm. of the substance were given by stomach tube after suspension in warm Liebig's extract solution and dissolving with the aid of enough sodium hydroxide to neutralize the acid.

The animal nucleic acid was prepared from the thymus gland according to the method of Jones (7). Analysis showed a composition of 14.5 per cent of nitrogen and 8.1 per cent of phosphorus. The biuret test showed a trace of protein. This preparation, too, was sufficiently pure for our purpose. Since it was already in the form of the sodium salt, it was dissolved in Liebig's extract solution and given by stomach tube quite warm to prevent gelatinization. 20 gm. were given in each experiment. This is an amount of nucleic acid contained in about 3 pounds of thymus, so that if nucleic acid has any effect on metabolism it should appear in the results.

The dogs were catheterized before administering the substance and after they came out of the calorimeter. A Kjeldahl analysis



of the urine of this period showed the amount of nitrogenous metabolism—protein, purine, and pyrimidine. The protein metabolism was established during the basal determinations. This was assumed to remain unaltered during the periods when the dogs were under the influence of the nucleic acids. Hence, by subtracting the nitrogen of the basal period from the nitrogen of the periods when nucleic acid was given the nucleic acid nitrogen was derived. From this figure a conception can be gathered

TABLE I.  
*Alcohol Checks in Relation to the Experiments.*

Date	Experiment No.	Time	R Q	Calories.		Remarks
				Indirect	Direct	
1922						
Apr. 4	152	3	0 667	63 44	63 43	Basal, Dog 18. Yeast nucleic acid, Dog 18.
" 5	94					
" 6	95					
" 10	153	3	0 670	61 25	64 89	Basal, Dog 19. Yeast nucleic acid, Dog 19. Thymus " " " 19. Yeast " " " 19.
" 11	80					
" 12	81					
" 13	82					
" 18	83					
" 19	154	3	0 663	64 16	65 54	Basal, Dog 19 " " 19. Thymus nucleic acid, Dog 19.
" 22	155	2	0 666	42 92	43 25	
" 24	85					
" 25	86					
" 26	87					
" 27	156	2	0 661	41 32	41 77	
Average.....			0 665+	54 62	55 78	

of the amount of this substance metabolized during the experiment.

The calorimetric procedure was that employed in the other experiments on animal calorimetry reported in this Journal since 1912 by Professor Graham Lusk and his pupils.

It will be seen from the alcohol checks recorded in Table I that in a period of 3 weeks the respiratory quotients varied from 0.670 to 0.661, showing an average of 0.665+. Such an agreement gives complete reliability to the calculation of the indirect

TABLE II.  
*The Influence of the Nucleic Acids on Heat Production.\**

Dog No.	Experiment No.	Time	Calories		Remarks.
			Indirect	Direct	
18	94	hrs 1	15 67	15 97	Basal.
		2	16.53	18 32	
		3	16.61	17 67	
Total		. .	48 81	51 96	
18	95	1	15.14	16 64	Received 20 gm. yeast nucleic acid.
		2	15 26	17 63	
		3	16 39	17 70	
Total.		. . .	46 79	51 97	
19	80	1	17.81	19 11	Basal.
		2	17 97	19 72	
		3	19 23	20 32	
	85	1	17 16	17 86	Basal.
		2	19 98	18 12	
		3	20 64	19 95	
	86	1	18 51	17 58	Basal.
		2	19 03	18 69	
		3	20 01	19 85	
Total for 9 hrs			170 34	171 20	
" " 6 "			113 56	114 14	
19	81	1	18 19	18 23	Received 20 gm. yeast nucleic acid.
		2	19 08	19 43	
		3	19 10	18 28	
	83	4	18 81	16 76	
		5	17 81	16 83	
		6	19 36	20 72	
Total for 6 hrs			112 35	110 25	
19	82	1	18 13	18 76	Received 20 gm thymus nucleic acid.
		2	19 35	18 51	
		3	19 09	20 17	
	87	4	18 04	17 89	
		5	19 18	20 36	
		6	21 55	19 85	
Total for 6 hrs			115.34	115 54	

\* On account of their bulk, the detailed tables of these experiments are omitted.

heat. Moreover, the difference between the average direct and indirect heat was less than 2 per cent.

In Table II is given a summary of the animal experiments. Dog 18 failed to show any rise in heat production for 3 hours after the administration of 20 gm. of yeast nucleic acid (Experiments 94 and 95). This is confirmed in Dog 19, whose basal heat production for 6 hours was 113.56 calories (Experiments 80, 85, and 86), and after the ingestion of 20 gm. of yeast nucleic acid it was 112.35 calories for a similar period (Experiments 81 and 83). Dog 19 also failed to show more than a negligible elevation in heat production after the administration of thymus

TABLE III  
*Urinary Nitrogen after Giving Nucleic Acid in Liebig's Extract.*

Experiment No	Time	Control N	After nucleic acid	Nucleic acid N	Remarks
	hrs	gm	gm	gm	
	1-2	0 386	0 430	0 044	Received 20 gm thymus nucleic acid.
	3-4	0 298	0 402	0 104	
	5-6	0 256	0 360	0 104	
	6			0 252	
95	3	0 360	0 510	0 150	Received 20 gm yeast nucleic acid.
81	3	0 345	0 645	0 300	" 20 " " " "
83	3	0 354	0 483	0 129	" 20 " " " "
82	3	0 345	0 501	0 156	" 20 " thymus " "
87	3	0 354	0 414	0 060	" 20 " " " "

nucleic acid (Experiments 82 and 87). Thus, for 6 hours after the ingestion of 20 gm. of thymus nucleic acid the indirect heat was 115.34 calories. This is less than 2 calories above the basal, which for a period of that length is of no significance. Indeed, during the first 5 of the 6 hours the heat production was identical with the basal.

Before interpreting these results it is necessary to consider the amount of nucleic acid actually metabolized during the experimental periods. A 2 hour fractionation of the urine after the ingestion of 20 gm. of thymus nucleic acid by Dog 19 is shown in Table III. During 6 hours 0.252 gm. of "extra" nitrogen, attributable to nucleic acid metabolism, was eliminated in the

urine. On the basis of 14.5 per cent of nitrogen in the nucleic acid molecule, it would appear that somewhat less than 2 gm. of nucleic acid were metabolized in that time. Only in Experiment 87 did the nucleic acid metabolism fall below this level. In the other experiments it was considerably higher. This is several times the nucleic acid metabolism of a normal dog, for Benedict's Dalmatian dog (8) excreted 0.200 gm. of purine nitrogen in 24 hours. These experiments may, therefore, be considered an efficient test of whether the nucleic acids in their metabolism influence the heat production. In view of the fact that, following the administration of nucleic acids, their metabolism begins to fall off after the 8th hour (9), no delayed effect on the heat production need likely be anticipated.

In calculating the indirect calories no heat value was given to the hourly nucleic acid nitrogen. It would be difficult to assign any, since the physiological heat value of a gram of nucleic acid, in the present state of knowledge with regard to their fate, is highly problematical. If we tentatively accept the value derived in the note earlier in this paper—about 2 calories to the gram of nucleic acid—the 2 gm. of this substance metabolized during 6 hours in these experiments would not alter the hourly indirect heat very significantly, hence the usual method of calculating the indirect heat, which was employed in these experiments, is probably adequate. Moreover, the good agreement between the average direct heat and the indirect lends support to this probability.

#### SUMMARY.

1. 20 gm. of yeast and thymus nucleic acids were given to dogs in order to promote an increased metabolism of these substances and to study the effect on the basal metabolism.

2. The heat production of these dogs remained practically at the basal level for 6 hours after the administration of the nucleic acids.

3. The nucleic acids exert no specific dynamic effect.

We wish to acknowledge our gratitude to Professor Graham Lusk for his advice and permission to use his trained Dogs 18 and 19. Also, we wish to thank Mr. James Evenden for assistance during the calorimeter experiments.

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## THE INFLUENCE OF INSULIN ON PHLORHIZIN DIABETES.

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This study was undertaken with the point in view of winning fresh data on the mechanism of phlorhizin diabetes and in the hope that such data would give ground for a direct method of assaying insulin, in terms of glucose utilized.

A completely phlorhizinized animal suffers from two essential incapacities. One, the inability to utilize sugar, from which defect follows the familiar train of pathological findings that constitutes the picture of true diabetes. This is well established. A. I. Ringer (1) demonstrated that as much as 75 gm. of ingested glucose can be quantitatively recovered in the urine of a completely phlorhizinized dog, and Lusk (2) found that the ingestion of this large quantity did not change the respiratory quotient from the diabetic level during a period of 4 hours.

The other defect relates to the inability of the kidneys to resorb the sugar they filter from the blood, resulting in a persistent glucosuria and the hypoglucemia which distinguishes phlorhizin from pancreatic diabetes. This much lowered renal threshold for sugar has somewhat confused the essential diabetic character of the phlorhizin injury. Indeed, it is held in many quarters that the failure of the phlorhizinized body to metabolize glucose is due to the subnormal blood sugar induced by the kidney leak. One cannot drink water from a sieve. Recently Nash and Benedict (3) have put the quietus on this view by giving glucose in sufficient amounts to induce protracted hyperglucemia in phlorhizinized dogs and recovering it quantitatively in the urine. That phlorhizin produces a real lesion of the sugar-burning mechanism can no longer be held as problematical.

Whence does this incapacity on the part of the phlorhizinized organism to metabolize sugar arise? Nash and Benedict postulate:

That phlorhizin forms a relatively stable union with the "carbohydrate receptors" of the cells, with the result that sugar molecules can no longer enter into the hypothetical combination prerequisite to their combustion. We conceive that such a combination between phlorhizin and the carbohydrate receptors of kidney cells may account for their failure to retain sugar or to reabsorb it from urine. The phlorhizin-cell union is presumably considerably more stable than the corresponding dextrose combination. According to this view, the sugar starvation in phlorhizin diabetes is analogous to the oxygen starvation in carbon monoxide poisoning.

It seems unlikely that a gram of phlorhizin, administered daily for 3 days, would effectually blockade the vast frontier of sugar-metabolizing cells of a large dog. It seems more reasonable to assume that some limited vital junction in the sugar-utilizing mechanism is attacked. Such a point is obviously the pancreas. Does phlorhizin affect the pancreas by temporarily abolishing its internal secretion? If it does, then the phlorhizin diabetic syndrome is accountable.

Insulin, the active antidiabetic hormone successfully extracted from the pancreas by Banting and his coworkers (4), is a drug with which we can put this hypothesis to a critical test. If the administration of insulin to a completely phlorhizinized dog causes that animal to utilize sugar, by the standard criteria, as it does in a depancreatized dog or in the human diabetic, then the idea of an essential failure of the body cells (in the sense of Nash and Benedict) to metabolize glucose can no longer be maintained. It would be strongly indicative of a phlorhizin injury to the pancreas, preventing the production of the antidiabetic hormone.

The following experiments direct one to this belief.

#### *General Procedure.*

Female dogs between 9 and 13 kilos in weight were used. These were fasted throughout the experimental period. On the 3rd day the subcutaneous injection of 1 gm. of recrystallized phlorhizin, suspended in olive oil, was begun and repeated every 24 hours until the end of the experiment. On the 3rd day of

phlorhization the urine was collected in 12 hour samples and analyzed as the preliminary period. The night sample usually showed that the animal was completely diabetic. On the 4th day the influence of insulin was studied, and the 5th day constituted the after period.

The insulin used was the Eli Lilly and Company preparation of 5, 10, or 20 units per cc. of strength, as indicated in the protocols. It was carefully measured and injected subcutaneously. The sugar given was Merck's pure dextrose. The dogs in their hunger usually lapped up the sweet solution quantitatively. Urinary nitrogen was determined by the Kjeldahl method; sugar by the Benedict method; blood sugar by the modified Benedict-Lewis method; and the ketone bodies were tested by the sodium nitroprusside and ferric chloride tests. The calorimeter experiments were done in the calorimeter employed by Lusk (5) in the experiments on Animal Calorimetry reported in this Journal during the last 12 years.

#### EXPERIMENTAL.

Early in the experiments a phlorhizinized dog was given 1 cc. of insulin containing 10 units, and 3 hours later the animal went into coma and convulsions. This was interpreted as the hypoglycemic syndrome attending the use of insulin, as described by Banting and his coworkers (6). This supposition was confirmed by the fact that the administration of glucose by mouth and vein brought the animal back to its former state in a few hours. In view of the low blood sugar of the dogs before the insulin injections, it was realized that these clamant symptoms of hypoglycemia would invariably terminate the experiment before any data of value could be gathered. Hence, thereafter a sufficient quantity of glucose was given with the insulin to overcome the excessive hypoglycemia.

*Effect of Insulin on Blood Sugar.*—Inspection of Table I will reveal that insulin in these phlorhizinized dogs prevents the development of the hyperglycemia which ordinarily follows the ingestion of glucose (3). Moreover, it actually reduces the blood sugar below the low level from which the experiments began and the effect lasts from 6 to 12 hours.



*Effect of Insulin on the Fate of Glucose.*—Tables II and IV record the data on this phase. Dog 11 received 40 gm. of glucose during complete phlorhization and eliminated in the urine, during the next 12 hours, 36 gm. of glucose above the basal output. 90 per cent of the total amount ingested was thus recovered in the urine. The 10 per cent loss may be regarded as a liberal experimental error and will be taken into account in all subse-

TABLE I.  
*Influence of Insulin on the Blood Sugar of Phlorhizinized Dogs.*

Dog No	Time	Blood sugar per 100 cc	Remarks
		mg.	
1	10 a m.	65	10 gm glucose and 5 units insulin at 10 a.m.
	1 p m.	57	
7	9 a m.	62	10 gm. glucose and 10 units insulin at 9 a m.
	11 "	58	
	1 p m.	40	
	3 "	48	
	5 "	62	
	7 "	62	
8	9 a m.	72	10 gm. glucose and 10 units insulin at 9 a m.
	11 "	60	
	1 p.m.	46	
	3 "	40	
	5 "	48	
10	10 a.m.	77	15 gm glucose and 20 units insulin at 10 a m.
	1 p m	100	
	5 "	47	
	10 "	53	

quent calculations. This experiment will serve as the control and will show the method of calculation.

The average D:N ratio of the fore and after periods was 3.5. During the experimental 12 hour period 4.5 gm. of nitrogen were eliminated. On the basis of the average D:N ratio, 15.8 gm. of glucose ( $3.5 \times 4.5$ ) derived from the metabolism of protein should have been excreted in the urine. But 51.2 gm. were actually recovered, hence an excess of 36.4 gm. resulted from

the ingestion of 40 gm. of glucose. This is what is known as "extra glucose" elimination.

The next day Dog 11 received 40 gm. of glucose again, but was simultaneously given 1 cc. of insulin containing 20 units. Table IV shows that only 23.8 gm. of extra glucose were recovered in the next 12 hours. *Under the influence of 20 units of insulin 12.2 gm. of glucose disappeared.* What happened to it? Was sugar oxidized?

The dog was in the calorimeter during the first 5 of the 12 hours. Table VII gives the data. The respiratory quotient rose maximally to 0.75 from a basal of 0.71. This means that the body was oxidizing a little over 0.5 gm. of sugar per hour, which can account for 6 of the 12 gm. of glucose that were assimilated. The other 6 gm. might be conceived as having been deposited as glycogen. Cori, Cori, and Pucher (7) have recently shown by direct analysis that normal animals under the influence of insulin deposit glycogen in the liver, simultaneously with the reduction in the amount of glucose in the blood and tissues. The glycogen starvation of the diabetic organism would favor such an early deposition.

But on the whole the assimilation of 12 gm. of glucose is a small effect after the injection of 20 units of insulin. This particular lot of insulin had been kept in the ice box for almost a month. It is possible that it had deteriorated on standing and lost its strength. Accordingly, a fresh lot was employed the following day when the same dog received 20 gm. of glucose with 20 units of insulin, and 2 hours later 40 gm. of glucose and 10 additional units of insulin. Then it was put in the calorimeter and the metabolism of the next 4 hours studied; that is, the metabolism of the 4th, 5th, 6th, and 7th hours after insulin at a time when a large amount of glucose was in circulation and the insulin had developed its maximal effectiveness.

The results are seen in the data of Tables VI and VII. The respiratory quotient rose to a maximum of 0.84. This shows unmistakably a good combustion of glucose. During each hour of this period the non-protein calories were about 18. A respiratory quotient of 0.84 means that 9 of these calories came from the combustion of glucose. *This maximally phlorhizinized dog oxidized over 2 gm. of glucose per hour.*

TABLE II  
*Influence of Insulin on the Elimination of Glucose by Phlorhizinized Dogs.*

Dog No	Period.	Urne.						Remarks.
		Ketone bodies						
		D	N	D	N	D	N	
	12 hrs.	gm	gm					
11	Fore.	21 4	5 91	3 61				Received 40 gm. glucose.
	First 12 hrs.	51 2	4 52					
	Second 12 "	17 5	5 10	3 43				
	Fore.	17 5	5 10	3 43				
	First 12 hrs.	35 6	3 43					40 gm glucose and 20 units insulin.
	Second 12 "	16 5	3 44	4 80				
	Fore	10 6	3 32	3 2				37 gm glucose and 10 units insulin.
	First 12 hrs	28 5	1 96				Strongly positive.	
Second 12 "	11 0	2 72	4 0			Positive		
1	Thrd 12 "	10 4	2 95	3 5			Strongly positive.	
	Fore.	23 7	6 86	3 45				10 gm. glucose and 5 units insulin.
	"	24 8	6 62	3 74			10 " " 5 "	
	First 12 hrs.	24 3	5 14					
Second 12 "	23 2	5 10						
3	Fore.	11 2	3 06	3 66			Strongly positive.	20 gm. glucose and 10 units insulin.
	First 12 hrs.	16 3	2 38				0	
	Second 12 "	13 7	2 87	4 80			Positive.	
	Thrd 12 "	12 0	3 49	3 40			Strongly positive.	

12	Fore. First 12 hrs Second 12 "	11 4 15 2 11 2	4 04 2 15 1 5	2 80 7 0	Strongly positive. 0 Trace.	15 units insulin and 30 gm. glucose 2 hrs. later.
5	Fore. First 12 hrs. Second 12 " Fore. First 12 hrs. Second 12 "	11 0 21 4 13 5 13 5 17 5 11 0	3 03 6 42 4 12 4 12 6 1 2 8	3 63 3 34 3 29 3 29 2 87 3 9	Strongly positive. Trace. Strongly positive.	200 gm. meat.  200 gm meat and 10 units insulin.
2	Fore. " First 12 hrs. Second 12 "	14 2 14 9 12 0 16 8	3 72 3 69 2 59 2 86	3 82 4 03 4 63 5 9		10 units insulin at start; 5 units 4 hrs. later; 5 units 8 hrs. later.

Somewhat less direct evidence of the utilization of glucose during the three corresponding periods in Dog 11, under discussion, is gathered from Table III.

It will be seen that in the last period, when the best conditions were observed, the extra glucose eliminated was cut in half, as compared with the control. This confirms the calorimetric evidence of glucose utilization, or *vice versa* it may henceforth be judged that a fall of the extra glucose from the maximal possibilities in these experiments means a *real* utilization of sugar.

In the other experiments recorded in Tables II and IV glucose was given in varying amounts and insulin in varying doses. In each case a large percentage (from 30 to 65 per cent) of the glucose ingested was assimilated during the first 12 hours. During the second 12 hours the D:N ratio, instead of returning to the

TABLE III

Day.	Time	Treatment	Extra glucose
	<i>hrs</i>		<i>gm</i>
3	5	40 gm. glucose; no insulin.	28 2
4	5	40 " " 20 units insulin.	22 0
5	5	60 " " 30 " "	13 5

level of the fore periods, as it did in the control dog (No. 11, Table II), remained elevated, indicating a further extra glucose elimination. What is the significance of this additional elimination of glucose in the second 12 hours?

Let us take a specific example—Dog 12. A glance at Table II shows that the sugar of the fore period is 11.4 gm. and the nitrogen 4.04 gm., giving a fore period D:N ratio of 2.8. In the second 12 hours after the combined administration of glucose and insulin the sugar returns to 11.2 gm., but the nitrogen, due to the depression of the protein metabolism has fallen to 1.5 gm., giving a D:N ratio of 7. The sugar resulting from the protein metabolism is, therefore, only 4 gm. ( $2.8 \times 1.5$ ) and the extra glucose 7 gm. Where does this considerable extra glucose come from? There was no sugar administered during that period. The fall in blood sugar cannot account for it, because the maxi-

mal fall in these dogs is from 65 mg. per 100 cc. to 45 mg. (the convulsive level), and this would yield less than 2 gm. of sugar in a 10 kilo dog if all of it were excreted. Very likely the extra glucose in question represents the quantity held over from the preceding period. It seems reasonable to suppose that, if these extra 7 gm. of glucose were circulating in a free state, in excess, during the experimental first 12 hours, they would probably have been excreted by the permeable kidneys. Since they were not eliminated until the next 12 hours, it suggests that they were held in combination, probably as glycogen, under the influence of insulin. When that restraining influence wore off

TABLE IV.  
*Influence of Insulin on the Oxidation of Glucose.*

Dog No	Glucose ingested *	Extra glucose first 12 hrs	Glucose assimilated	Extra glucose second 12 hrs	Glucose oxidized	Units insulin injected	Glucose oxidized per unit.
	gm	gm	gm	gm	gm		gm
11	36 0	23 8	12 2	4 7	7 5	20	0 38†
4	33 3	21 9	11 4	1 9	9 5	10	0 95
3	18 0	7 8	10 2	3 4	6 8	10	0 68
12	27 0	9 2	18 8	7 0	11 8	15	0 80
1	9 0	5 3	3 7	0	3 7	5	0.74
5	34.9	28 5	6 4	0	6 4	10	0 64

\* From the amount of glucose actually ingested 10 per cent was deducted to allow for the experimental error in the recovery of glucose in the urine.

† The low value in Dog 11 may be questioned because of the probable deterioration of the lot of insulin used, as is mentioned earlier in the paper.

in the second 12 hours the leaking kidneys took their toll and the deposited glycogen was eliminated. It is well known that phlorhizin is an excellent deglycogenating agent.

If we accept this view, that the extra glucose of the second 12 hour period represents that part of the glucose, assimilated in the first 12 hours, which was stored as glycogen, the difference between the two will indicate the amount of glucose that was actually utilized or oxidized. From these data it may be discovered how much glucose a unit of insulin will cause to be oxidized. Table IV is constructed to bring out this phase of the experiments.

*Effect of Insulin on Protein Metabolism.*—The protein metabolism of a phlorhizinized dog is high. Table V, which records the amount of protein metabolized in the experiments, shows that during the 12 hour fore periods the dogs broke down from 25 to 35 gm. of protein; that is, from two to four times the normal.

A. I. Ringer (1) has called attention to the fact that glucose given to a phlorhizinized animal, although it is not burned, lowers the high protein metabolism. In the present series of experiments glucose was simultaneously given with insulin. It is, therefore, necessary to bear in mind that the sugar itself has a sparing action on protein metabolism, and to allow for this effect in interpreting the influence of insulin.

It is obvious that, if insulin causes the oxidation of glucose (which has already been demonstrated), it restores a source of calories to the body and hence the emergency call on the proteins may be withdrawn, so that a sparing action on protein metabolism may reasonably be anticipated.

A glance at Table II reveals at once that after the administration of insulin the urinary nitrogen of the succeeding 12 hour periods falls. This indicates a sparing action on protein metabolism. But what part of this is due to the sugar administered and what part to the insulin alone?

Dog 2 (Table V), which was completely phlorhizinized and received no glucose, showed a reduction of protein metabolism of 30 per cent in the first 12 hours and 23 per cent in the second 12 hours, under the influence of 20 units of insulin. Dog 5 (Table V) was given 200 gm. of meat and metabolized 65 gm. of protein in 24 hours. The experiment was repeated the next day under the influence of 10 units of insulin, and the metabolism of protein fell 15 per cent, to 55 gm. The sparing effect on protein metabolism in these dogs is due to the insulin alone.

Dog 11 (Table V) received 40 gm. of glucose, which reduced the protein metabolism by 23 per cent in the first 12 hours and 12 per cent in the second. When the experiment was repeated under the influence of 20 units of insulin it fell 33 per cent in the first 12 hours and 33 per cent in the second 12 hours. Thus, insulin caused a greater and a more prolonged fall of protein metabolism than glucose alone.

This is better seen in Dog 12 (Table V). The protein metabolism during the first 12 hours fell 50 per cent and in the second 12 hours it fell 73 per cent. Indeed, in the latter period the protein metabolism was that of a normal dog. That is to say, insulin completely corrected the diabetes, as far as the protein metabolism was concerned.

To return to Dog 11, when the animal was in the calorimeter (Table VI), if we compare the 5 hour period when only glucose

TABLE V  
*Influence of Insulin on the Protein Metabolism of Phlorhizinized Dogs.*

Dog No	Protein metabolism (gm N $\times$ 6.25)			Remarks
	Fore period, 12 hrs	First 12 hrs	Second 12 hrs	
	gm	gm	gm	
2	23 13	16 20	17 9	20 units insulin.
5		40 10 38 10	25 7 17 5	200 gm meat. 200 " " and 10 units insulin.
11	36 90 31 9	28 30 21 40	31 9 21 5	40 gm glucose. 40 " " and 20 units insulin.
12	25 2	13 4	9 4	15 units insulin and 30 gm. glucose 2 hrs later.
	Fore period, 5 hrs	First 5 hrs		
11	18 0 18 0 18 0	11 8 10 3 5 8		40 gm. glucose. 40 " " and 20 units insulin. 20 units insulin and 40 gm glucose 2 hrs later.

was given with the 5 hour experimental periods when glucose and insulin were given, it will be seen (Table V) that in the last period, when the dog oxidized 2 gm. of glucose per hour, the protein metabolism was cut in half. It fell from 11.8 gm. of protein in 5 hours to 5.8 gm., or from 5 protein calories per hour to 2.5. This is a substantial and convincing reduction.

In Dogs 1, 3, and 4 there is also a reduction in the protein metabolism, but it is difficult to separate the effects of the glucose *per se* and the insulin.



*Effect of Insulin on the D:N Ratio.*—It may be supposed that, since insulin causes sugar to be oxidized, the D:N ratio would fall in the direction of zero. Insulin does this in depancreatized animals and in human diabetics. The tables, however, record the failure to depress the D:N ratio of phlorhizinized animals much below the ratio of the fore periods.

Dog 2 (Table II), which without glucose successfully survived 20 units of insulin (in divided doses) without developing convulsions, failed to show a lower D:N ratio. Thus, on the contrary, from an average fore period ratio of 3.9, it rose to 4.63 and 5.9.

Dog 5 (Table II) was given 200 gm. of meat as a control. The D:N ratio fell from an average of 3.46 ( $3.63 + 3.29 \div 2$ ) to 3.24. Such an amount of meat supplied enough glucose to prevent too great a hypoglycemia, when insulin was later introduced, and yet left the basal D:N ratio practically unchanged. It was a better test case than that of Dog 2, just mentioned. When 10 units were given with the 200 gm. of meat the D:N ratio fell to 2.87. Although this was not a very striking reduction, it indicates that the tendency was in the direction of zero.

In the other animals the simultaneous administration of glucose with insulin would, of course, veil a fall in the D:N ratio. Some indication of the depressing effect of insulin on the D:N ratio might, however, have been gathered from a comparison of D:N ratios of a period when glucose alone was given and when the experiment was repeated under the influence of insulin. Such a comparison may be made in Dog 11 (Table VI). On the 5th day 40 gm. of glucose were given and the D:N ratio was 18.3. On the 7th day during a similar period when glucose was surely oxidized, for the respiratory quotient was 0.82, the D:N ratios remained at 17.6. The failure of the D:N ratio to show a substantial fall is very likely only an arithmetical effect and not physiological, for it will be noted that the sugar output of the latter period was less than half of the former. It is only the concomitant fall in urinary nitrogen which kept the ratio elevated.

It would appear, then, that, contrary to expectation, the D:N ratio of phlorhizin diabetes is only insignificantly reduced below the level of the fore period by the administration of insulin.

This is not to be interpreted, however, as a failure on the part of that drug to correct the diabetes; *i.e.*, the inability to oxidize sugar. It is rather to be regarded as a failure on the part of insulin to overcome the low renal threshold of the phlorhizinized state, which permits an amount of blood sugar to leak out constantly and necessitates a constant catabolism of protein to supply that loss. Since the sugar and the nitrogen coming from this protein both ultimately appear in the urine, the relation between them must inevitably be 3.6 to 1. This would be so even if ingested sugar were oxidized. That is probably why the D:N ratio fails to fall in these experiments.

*Effect of Insulin on Ketonuria.*—It will be noticed in Table II that in the 12 hour period when the insulin was administered, acetone disappeared from the urine. The test for this ketone was negative. During the 12 hours following, a faint trace reappeared as indicated by the color. During the fore period the test was always strongly positive. This would appear to confirm the other evidence for the combustion of glucose. Presumably the antiketogenetic effect of the insulin is due to the complete oxidation of the fatty acids made possible by the combustion of glucose.

*Effect on Heat Production.*—Dog 11, which was the animal studied in the calorimeter, has a basal metabolism of about 16 calories per hour and shows an hourly protein metabolism of between 0.6 and 0.7 gm. Under phlorhizin, as may be seen in Tables VI and VII, the basal metabolism was 23 calories per hour, and the protein metabolism was elevated to 2.9 gm. per hour. The higher basal metabolism is usually attributed to the specific dynamic effect of the quadrupled protein metabolism.

When 40 gm. of glucose were given the heat production remained unchanged, although the protein metabolism fell somewhat, to 2.4 gm. per hour. In the last experiment, when glucose was given at the height of insulin activity and 2 gm. of glucose were oxidized per hour, as has already been observed, the protein metabolism fell to one-half of the fore period basal; that is, to about 1.2 gm. of protein per hour. This fall should, therefore, have produced a perceptible reduction in heat production. The experiment bears this out, because the hourly calories fell from 23.3 to 21.2, a decrease of 2 per hour.

TABLE VI  
*Influence of Insulin on the Metabolism of a Phlorhizinized Dog (No. 11).*

Condition.	Time.	Treatment	D	N	D N	R.Q.	Calories per hr.		
							Protein.	Indirect.	Direct.
4th day of phlorhizin.	hrs 5	Basal metabolism. Received 40 gm. glucose. " 40 " " and 20 units insulin simultaneously.*	gm 9 1	gm 2 29	3 98	0 700	6 0	23.3	21.2
5th day of phlorhizin.	Next 5.		8 7	2 40	3 63				
6th " "	5		34 7	1 90	18 3	0 711	5.0	23.3	23.2
7th day of phlorhizin.	5	Received 20 gm. glucose and 20 units insulin. 3 hrs later 40 gm. glucose and 10 units insulin.	27 8	1 65	17 0	0 763	4 0	23.3	22 0
			16 6	0 94	17 6	0 823	2 5	21.2	21.4

\* This lot of insulin (referred to in the text) was over a month old and had probably deteriorated.

The alcohol check (Table VII) at the beginning of the experimental period, showed a respiratory quotient of 0.666 (theoretical 0.667). We may, therefore, put full reliance on the indirect heat, which was indeed strikingly constant, above 23 calories throughout the preliminary experiments, Nos. 150 and 151. In Experiment 152 the fall of 2 calories was maintained during 4 successive hours, so that no doubt about the reliability of the data can be raised.

*Use of a Phlorhizinized Dog in Assaying Insulin.*

The method of assaying insulin at present in common use, as devised by Banting and his coworkers (8), confines itself to ascertaining the number of units contained in each cubic centimeter of insulin. The unit is an arbitrary quantity. It is defined by its power to reduce the blood sugar of rabbits of standard weight to the toxic level. It would be much better to equate the unit in terms of actual grams of sugar oxidized. This would put the use of insulin on a definite mathematical basis.

The ideal animal for such a standardization would naturally be a depancreatized dog. But obviously the use of such an animal is impractical because of the involved surgical procedure. It appears from the data presented above that a phlorhizinized dog might be used effectively as a substitute for a depancreatized animal for the purpose of assaying insulin directly in terms of glucose utilized. The proposal rests on the facts that dogs can be rendered completely diabetic with very little effort by means of phlorhizin injections, and that insulin causes these animals to oxidize glucose. Whether the extent to which insulin causes the oxidation of glucose in these dogs is comparable to its influence in man is still to be worked out on a large scale. Woodyatt (9) reports that, by studies on carefully selected patients, he obtained a maximal utilization of 1 to 1.5 gm. of glucose per unit of insulin. It will be seen from Table IV that in the present experiments the values fall between 0.65 and 0.95 gm. to the unit.

In this paper glucose utilized is held strictly to mean glucose oxidized. It is not clear from his paper whether Woodyatt restricts the meaning of utilization to this sense or whether glucose deposited as glycogen is also regarded as glucose utilized.

TABLE VII—Dog 11 (*Lusk's*)

Date.	Experiment No	Time.	CO <sub>2</sub>	O <sub>2</sub>	R Q	H <sub>2</sub> O	Urine N	Non-protein.			Calories.				
								CO <sub>2</sub>	O <sub>2</sub>	R.Q.	Protein	Non-protein	Total calcu- lated.	Total found.	
1923															
May 27	175	12 n.- 1 p.m.	5 49	5 96	0 670	4 48								21.17	20.20
		1 p.m.-2 "	6 21	6 83	0 661	4 72								23.57	23.15
					0 666									22.37	21.68
May 23	149	12 n.- 1 p.m.	6 78	6 92	0 713	10 16	0 46	4 94	4 82	0.745	6 0	15 97	21 97	21.67	
		1 p.m.-2 "	7 21	7 27	0 710	9 60	0 46	5 37	5 17	0.755	6.0	17.18	23.18	20.62	
		2 " -3 "	7 58	7 75	0 711	9 30	0 46	5 74	5 65	0.739	6 0	18 69	24 69	21.39	
					0 711									23 28	21.23
May 24	150	12 n.- 1 p.m.	7 15	7 25	0 717	9 97	0 38	5 63	5 52	0.742	4 96	18 27	23 23	22.99	
		1 p.m.-2 "	7 22	7 32	0 717	9 52	0 38	5.70	5 59	0.741	4 96	18 50	23 46	23.31	
		2 " -3 "	7 50	7 81	0 698	11 73	0.38	5 98	6 08	0.699	4.96	19 44	24 90*	26.67*	
					0 711									23 34	23 15
May 25	151	12 n.- 1 p.m.	7 04	6 95	0 737	9 55	0 31	5 80	5 54	0.761	4 04	18 43	22 47	20.94	
		1 p.m.-2 "	7 20	7 33	0 714	9 01	0 31	5 96	5 92	0.732	4 04	19 54	23 58	22 93	
		2 " -3 "	7 44	7 21	0 751	8 95	0 31	6 20	5 80	0.777	4 04	19 38	23.42	22.28	
		3 " -4 "	7 45	7 29	0 743	8 52	0 31	6 21	5 88	0.768	4 04	19 60	23.64	22.78	
					0 736									23 28	22.23
May 26	152	12 n.- 1 p.m.	7 26	6 46	0 817	9 82	0.188	6 51	5 61	0.844	2 49	19.05	21.54	22.84	
		1 p.m.-2 "	7 21	6 47	0 811	9.01	0.188	6 46	5.62	0.836	2 49	19 09	21 58	21.01	
		2 " -3 "	7 07	6 23	0 823	8 22	0.188	6 32	5 38	0.854	2.49	18 29	20.78	20.30	
		3 " -4 "	7 16	6.22	0 837	8 05	0.188	6 41	5 37	0.868	2.49	18.36	20 85	21.53	
					0 823									21.19	21.42

\* Third hour omitted in average.

*Calorimeter Dog 18).*

Body temperature.			Morning weight. kg	Behavior of dog.	Food.
Start.	End.	Difference.			
				Alcohol check.	
38.15	38.27	0.12	10	Very quiet.	Fasting; 4th day of phlorhizin.
38.27	38.21	0.06		" "	
38.21	38.17	0.04		" "	
38.17	38.20	0.03	9.6	Very quiet.	40 gm. glucose 1 hr. before; 5th day of phlorhizin.
38.20	38.30	0.10		" "	
38.30	38.43	0.13		Urinated in box	
38.31	38.23	0.08	9.4	Very quiet.	40 gm. glucose and 20 units insulin 1 hr. before.
38.23	38.34	0.11		" "	
38.34	38.43	0.09		" "	
38.43	38.54	0.11		" "	
38.57	38.70	0.13	9.3	Very quiet.	20 gm. glucose and 20 units insulin 3 hrs. before. 40 gm. glucose and 12 units insulin 1 hr. before.
38.70	38.68	0.02		" "	
38.68	38.66	0.02		" "	
38.66	38.71	0.05		" "	

In the broader definition the values derived from these experiments, as is seen from the third column in Table IV, are comparable to Woodyatt's figures.

#### SUMMARY.

1. Insulin injected in completely phlorhizinized dogs results in an oxidation of glucose.

2. A maximal oxidation of 0.95 gm. of glucose per unit of insulin was obtained.

3. That the glucose was really oxidized is shown by the respiratory quotient, which rose maximally to 0.84, indicating a combustion of 2 gm. of glucose per hour.

4. Evidence is adduced to show that glucose was also stored as glycogen.

5. Insulin reduced the protein metabolism from its high diabetic level.

6. Insulin caused the disappearance of ketone bodies.

7. Insulin reduced the heat production from the high diabetic level by 2 calories per hour.

8. The bearing of these data on the mechanism of phlorhizin diabetes is discussed. The argument is brought forth that the striking effect of insulin, in correcting the diabetes of a phlorhizinized dog, indicates that phlorhizin transitorily injures the pancreas by preventing the production of the antidiabetic hormone.

9. The use of phlorhizinized dogs is proposed in assaying insulin directly in terms of glucose utilized.

I must here acknowledge with thanks my debt to Professor Graham Lusk for his courtesy in lending me his highly trained calorimeter Dog 19, for permission to use his calorimeter, and for valuable criticism in the interpretation of the data. I also wish to thank Mr. James Evenden for assistance during the calorimeter experiments.

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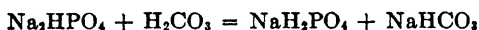
# THE EFFECT OF INJECTIONS OF SODIUM PHOSPHATES AND SODIUM HIPPURATE UPON THE EXCRETION OF ACID AND AMMONIA BY THE KIDNEY.

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Cushny (1) has shown that injections of disodium phosphate in dogs cause an increase in the titratable acidity of the urine, but the exact relation of the amount of salt injected to the acid excreted was not determined. This shows that the kidney can retain at least a portion of the base from the disodium phosphate. The ultimate change may be represented by the following equation.



He believes that one sodium ion of the disodium phosphate is taken up by the epithelial cells as the urine passes through the tubules. His work does not really show what quantity of sodium is retained by this mechanism. The experiments to which we have just referred, did not yield such information.

Both Cushny (1) and Rüdell (2) show that such substances as glucose, sodium chloride, and sodium sulfate, really decrease the amount of titratable acidity in the urine. The urine, after the injection of any of the substances mentioned above, may have the same pH as that of the blood plasma. This, according to Cushny, is due to the diuresis brought about by the substances injected. The diuresis causes such a rapid flow through the tubules that there is little exchange of ions between the liquid in the tubules and the cells lining them.

It is a well known fact that the ingestion of sodium salts of those organic acids which are oxidized, decrease the titratable acidity in the urine, having an effect very similar to sodium bicarbonate in this respect.

Henderson and Palmer (3) have shown that the hydrogen ion concentration of the urine is within the phosphate range under normal, as well as pathological, conditions. The amount of titratable acidity, according to them, increases with the increase in hydrogen ion concentration when the urine is titrated to pH 7.4. This, of course, follows inasmuch as practically all the acid of the urine is in the form of dihydrogen phosphate and the pH is a function of the ratio between mono- and dihydrogen phosphates. On the other hand, they find no very pronounced relation between the pH and the amount of ammonia excreted. Their results show some decline in ammonia with the increase in pH. It has long been known, however, that the amount of ammonia in the urine does increase when excessive amounts of acid are excreted as in severe cases of diabetes mellitus in man.

Marriott and Howland (4) found that the administration of monosodium phosphate by mouth to normal men, did not increase the amount of ammonia excreted by the kidney, while an equivalent amount of hydrochloric acid produced a considerable increase. Unless this increase in ammonia after the ingestion of hydrochloric acid is entirely of alimentary origin, such results are not explainable on any theoretical grounds known to the present writers. There seems to be no doubt that acids absorbed from the intestine are largely neutralized before or during the process of absorption, else the cells of the mucosa would be subjected to a hydrogen ion concentration which would certainly be fatal to their continued existence. It does not seem that even monosodium phosphate, which has a pH of about 4.5, could be absorbed without being previously buffered or neutralized in part. The effective change in the pH of the blood ought to be the same regardless of the nature of the acid ingested, providing the acids are not oxidized in the body.

On the other hand, it seems quite probable that a part of the hydrochloric acid would be neutralized by the ammonia resulting from the digestion and putrefaction of the protein in the alimentary tract. A portion, at least, of the ammonium chloride thus formed would be excreted by the kidney without being changed. Any sodium ammonium phosphate formed in the intestines after the ingestion of monosodium phosphate would undoubtedly be completely hydrolyzed in the blood stream and the ammonia

liberated would be transformed into urea by the liver or other tissues. The observation of Schittenhelm (5) that an increased formation of hydrochloric acid by the stomach is followed by a rise in the excretion of ammonia by the kidneys, supports the idea that a portion of urinary ammonia may be of alimentary origin.

Nash and Benedict (6) have reported experiments to support the idea that the ammonia of the urine is formed in the kidney. They found that the blood flowing away from the kidney contains more ammonia than that flowing into it. They are of the opinion that the amount of ammonia in the general circulation is entirely too small to supply all that is found in the urine, even though the kidney removed completely the ammonia from the blood. The increase in ammonia in the blood of the renal vein over that of the renal artery at least shows that the ammonium salts are not completely taken out of the blood by the kidney. These workers are not willing to admit that ammonia serves to neutralize acid in the blood and tissues, as was generally believed until recently. They ignore the alimentary tract as a source of this compound; perhaps they assume it to be completely transformed into urea by the liver and other tissues.

In the experiments reported in this paper, we have attempted to obtain data upon, first the quantity of sodium which the body can retain after the injection of the sodium salts of weak acids, and second the source of urinary ammonia.

#### EXPERIMENTAL.

The female dogs used in these experiments were allowed to fast until the ammonia and acid excretion became constant before injections were made. All injections were made subcutaneously, and usually two or more doses were given to each animal. The dogs were kept in large metabolism cages and catheterized twice a day. Special care was taken to prevent fermentation or putrefaction of the urine. The urine which happened to be voided in the cages was collected under toluene. It was found that if the cages were kept scrupulously clean, the urine voided while the animals were in the cages was not acted upon by microorganisms when preserved in this way. All results are reported in quantities excreted in 24 hours, but the ammonia and acid were determined twice a day on approximately 12 hour specimens of urine.

The ammonia was determined by the method of Folin (7), the acid was titrated with 0.1 N sodium hydroxide to a definite pink color of the phenolphthalein which was used as an indicator. The phosphates were titrated with standard uranium acetate solution, and the hippuric acid was determined by the method of Folin and Flanders (8). The  $\text{CO}_2$ -combining power of the whole blood was determined by the Van Slyke method.

#### *Experiments with Phosphates.*

The protocols of experiments on Dogs 3, 7, and 8 show results which agree with those of Cushny, indicating that disodium phosphate increases the amount of acid in the urine of dogs. In all these experiments, the increase in titratable acid plus ammonia is very nearly equivalent to the phosphate injected, showing that Cushny's reaction goes to completion; that is, if ammonia is to be considered as representing an equivalent amount of urinary acid.

In all three cases, the agreement was remarkably close. The percentage of ammonia to the total acid plus ammonia varies considerably from 29.55 to 77.21, but in all cases, the amount of ammonia was distinctly increased during the period of increased acid excretion. That sodium ions were being retained within the animal body is further shown by the increase in the alkali reserve during the period of increased acid excretion.

In two experiments (see Protocols IV and V) with monosodium phosphate on Dog 6, the results, as far as the urine is concerned, were very similar to those in which disodium phosphate was used. The proportion of ammonia to the total acid (titratable acid plus ammonia) was somewhat less, being 18.95 and 31.67 per cent, respectively. The average of these figures is 25.23 per cent, while in the experiments with disodium phosphate, the average percentage of ammonia to the total acid was 60.20. No importance can be assigned to this difference because the data are entirely too few for statistical comparisons.

It is to be noted that no metallic ions were lost to the body by the injection of monosodium phosphate, although it doubtless circulated in the blood for a time at least, largely in the form of disodium phosphate. The monosodium phosphate did not have any significant effect upon the  $\text{CO}_2$ -combining power of the blood,

## Protocol I.

Dog 3, weight 4 kilos.

Date.	Urine analyses.						Blood.	Period.
	Vol- ume.	0.1 N acid	NH <sub>3</sub>	P <sub>2</sub> O <sub>5</sub>	0.1 N total acid (acid + NH <sub>3</sub> )	0.1 M phos- phates.	CO <sub>2</sub>	
1923	cc	cc	gm	gm	cc	cc	vols per cent	
June 10	90	54	0.054	0.499	86	70	47.45	Normal.
" 11	125	16	0.144	0.590	101	83		"
" 11	Injected 1.4 gm. disodium phosphate at 6 p. m.							
" 12	110	122	0.088	0.852	176	120	51.57	Experimental.
" 12	Injected 1.4 gm. disodium phosphate at 6 p. m.							
" 13	125	80	0.191	1.250	192	176	57.90	Experimental.
" 14	115			0.348	80	49	55.39	Normal.

Injected equivalent of 197 cc. of 0.1 M disodium phosphate solution. Total acid recovered over control period is equivalent to 181 cc. of 0.1 N solution. Phosphate in excess over control period is equivalent to 143 cc. of 0.1 M solution. Ammonia in excess over control period is 0.091 gm. and is equivalent to 53.5 cc. of 0.1 N solution.

## Protocol II.

Dog 7, weight 3 kilos.

Date	Urine analyses						Blood	Period
	Vol- ume.	0.1 N acid	NH <sub>3</sub>	P <sub>2</sub> O <sub>5</sub>	0.1 N total acid (acid + NH <sub>3</sub> )	0.1 M phos- phates	CO <sub>2</sub>	
1923	cc	cc	gm	gm	cc.	cc	vols per cent	
July 3	75	60	0.150	0.277	147	39	43.57	Normal.
" 3	Injected 1.8 gm. disodium phosphate at 6 p. m.							
" 4	170	108	0.168	0.885	207	124	45.25	Experimental.
" 4	Injected 1.8 gm. disodium phosphate at 10 a.m.							
" 5	180	36	0.466	1.220	310	172	53.64	Experimental.
" 6	160	19	0.218	0.358	147	50	49.72	

Injected equivalent of 254 cc. of 0.1 M disodium phosphate solution. Total acid recovered over control period is equivalent to 260 cc. of 0.1 N solution. Phosphate in excess over control is equivalent to 218 cc. of 0.1 M solution. Ammonia in excess over control period is 0.327 gm. and is equivalent to 192 cc. of 0.1 N solution.

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## Protocol III.

Dog 8, weight 7 kilos.

Date.	Urine analyses.						Blood.	Period.
	Vol- ume.	0.1 N acid.	NH <sub>3</sub>	P <sub>2</sub> O <sub>5</sub>	0.1 N total acid (acid + NH <sub>3</sub> )	0.1 M phos- phates.	CO <sub>2</sub>	
1923	cc	cc	gm.	gm.	cc	cc.	vols per cent	
July 4	165	58	0 166	0 297	156	42		Normal.
" 5	110	59	0 135	0 242	138	34	42 38	"
" 5	Injected 3.0 gm. disodium phosphate at 6 p.m.							
" 6	215	126	0 220	1 740	255	245		Experimental.
" 6	Injected 3.0 gm. disodium phosphate at 9 a.m.; also at 6 p.m.							
" 7	390	116	0 681	2 810	523	396	42 10	Experimental.
" 8	430	71	0 380	0 730	203	103		"
" 9	Urine returned to normal.							

Injected equivalent of 635 cc. of 0.1 M disodium phosphate solution. Total acid recovered over control period is equivalent to 632 cc. of 0.1 N solution. Phosphate in excess over control period is equivalent to 630 cc. of 0.1 M solution. Ammonia in excess over control period is 0.830 gm. and is equivalent to 488 cc. of 0.1 N solution.

## Protocol IV.

Dog 6, weight 7.5 kilos.

Date.	Urine analyses						Blood	Period
	Vol- ume	0.1 N acid	NH <sub>3</sub>	P <sub>2</sub> O <sub>5</sub>	0.1 N total acid (acid + NH <sub>3</sub> )	0.1 M phos- phates	CO <sub>2</sub>	
1923	cc	cc	gm	gm	cc	cc	vols per cent	
June 19	75	106	0 0626	0 675	143	95		Normal.
" 20	285	86	0 0748	0 622	130	86	44 77	"
" 20	Injected 3.12 gm. monosodium phosphate at 6 p.m.							
" 21	65	243	0 0808	1 860	291	262	43 50	Experimental.
" 21	Injected 3.12 gm monosodium phosphate at 6 p.m.							
" 22	220	328	0 2230	2 310	469	362	43 36	Experimental.
" 23	150			0 930	202	131		

Injected equivalent of 520 cc. of 0.1 M monosodium phosphate solution. Total acid recovered over control period is equivalent to 487 cc. of 0.1 N solution. Phosphate in excess over control period is equivalent to 407 cc. of 0.1 M solution. Ammonia in excess over control period is 0.157 gm. and is equivalent to 92.5 cc. of 0.1 N solution.

## Protocol V.

Dog 6, weight 7.5 kilos (second experiment).

Date.	Urine analyses.						Blood.	Period
	Vol- ume	0.1 N acid	NH <sub>3</sub>	P <sub>2</sub> O <sub>5</sub>	0.1 N total acid (acid + NH <sub>3</sub> )	0.1 M phos- phates	CO <sub>2</sub>	
1923	cc.	cc.	gm	gm	cc	cc.	vols per cent	
June 27	530	73	0 328	0 477	266	67		Normal.
" 28	670	62	0 333	0 503	258	71	45 22	"
" 28	Injected 3.12 gm. monosodium phosphate at 4 p.m.							
" 29	410	182	0 632	1 710	555	241	44 40	Experimental.
" 29	Injected 3 12 gm monosodium phosphate at 9 a.m.; also at 4 p.m.							
" 30	590	352	0 342	3 240	553	457		Experimental.
July 1	335	49	0 241	0 435	191	61	44 95	Normal.

Injected equivalent of 778 cc of 0.1 M monosodium phosphate solution. Total acid recovered over control period is equivalent to 584 cc. of 0.1 N solution. Phosphate in excess over control period is equivalent to 559 cc. of 0.1 M solution. Ammonia in excess over control period is 0.313 gm. and is equivalent to 184 cc. of 0.1 N solution.

## Protocol VI.

Dog 5.

Date.	Urine analyses						Blood	Period
	Vol- ume	0.1 N acid	NH <sub>3</sub>	Sodium hippu- rate	0.1 N total acid (acid + NH <sub>3</sub> )	0.1 N hip- puric acid	CO <sub>2</sub>	
1923	cc.	cc	gm	gm.	cc	cc.	vols per cent	
June 19	95	97	0 0825		145			Normal.
" 20	95	124	0 0728		167			"
" 21	95	104	0 0885	.	163		45 35	"
" 21	Injected 2.5 gm. sodium hippurate at 6 p.m.							
" 22	120	136	0 2060	1 395	257	70	47 50	Experimental.
" 22	Injected 2.5 gm. sodium hippurate at 9 a.m.; also at 6 p.m.							
" 23	325	115	0 3570	4 750	325	237	48 12	Experimental.
" 23	Injected 2.5 gm. sodium hippurate at 11 a.m.							
" 24	275	55	0 374	2 210	275	110	48 70	Experimental.

Hippurate injected is 10 gm. which is equivalent to 496 cc. of 0.1 N solution. Total acid recovered over control period is equivalent to 417 cc. of 0.1 N solution. Hippurate in excess over control period is equivalent to 414 cc. of 0.1 N solution. Ammonia in excess over control period is 0.766 gm. and is equivalent to 398 cc. of 0.1 N solution.



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## Protocol VII.

Dog 6-A.

Date.	Urine analyses						Blood.	Period.
	Volume	0.1 N acid	NH <sub>3</sub>	Sodium hippurate	0.1 N total acid (acid + NH <sub>3</sub> )	0.1 N hippuric acid	CO <sub>2</sub>	
1923	cc	cc	gm.	gm	cc.	cc	vols per cent	
June 27	275	21	0 234		159			Normal.
" 28	155	39	0 260		192		44 32	"
" 28	Injected 2.5 gm. sodium hippurate at 4 p.m.							
" 29	195	50	0 531	2 24	362	112	50 76	Experimental.
" 29	Injected 2.5 gm. sodium hippurate at 10 a.m.; also at 6 p.m.							
" 30	175	97	0 202	4 24	215	211	52 10	Experimental.
July 1	140	35	0 205	0 434	168	21	55 95	"

Hippurate injected is 7.5 gm. and is equivalent to 371 cc. of 0.1 N solution. Total acid recovered over control period is equivalent to 362 cc. of 0.1 N solution. Hippurate in excess over control period is equivalent to 331 cc. of 0.1 N solution. Ammonia in excess over control period is 0.192 gm. and is equivalent to 111.7 cc. of 0.1 N solution.

## Protocol VIII.

Dog 12, weight 9.5 kilos.

Date	Urine analyses								Blood	Period.
	Volume	0.1 N acid	NH <sub>3</sub>	Sodium hippurate	P <sub>2</sub> O <sub>5</sub>	0.1 N total acid (acid + NH <sub>3</sub> )	0.1 M phosphates	0.1 N hippuric acid.	CO <sub>2</sub>	
1923	cc	cc	gm	gm.	gm	cc	cc	cc	vols per cent	
July 18	140	67	0 243		1 34	210	189			Normal.
" 19	120	105	0 216		1 06	232	150		42 67	"
" 19	Injected 2.55 gm. sodium hippurate at 3 p.m.									
" 20	110	134	0 300	2 44	0 59	310	84	122	46 23	Experimental.
" 20	Injected 3.00 gm sodium hippurate at 9 a.m.									
" 21	165	165	0 337	2 78	0 51	363	72	139	44 33	Experimental.

Hippurate injected is 5.55 gm. and is equivalent to 275 cc. of 0.1 N solution. Total acid recovered is equivalent to 231 cc. of 0.1 N solution. Hippurate in excess over control period is equivalent to 261 cc. of 0.1 N solution. Ammonia in excess over control period is 0.178 gm. and is equivalent to 105 cc. of 0.1 N solution.

or, any marked effect the injection may have had disappeared before the blood was drawn for the determination.

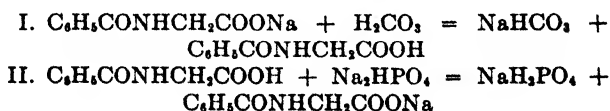
It is to be noted that the increase in phosphate excretion agrees quite well with the amount injected and in virtually all cases, the excretion of acid, ammonia, and phosphate had returned to normal on or before the 3rd day after the injection.

The injections of sodium hippurate as shown by the experiments on Dogs 5, 12, and 6-A had essentially the same effect upon the urine and blood as disodium phosphate under the same conditions. There may be a slightly less close agreement between the amount of salt injected and the total acid excreted, but this difference may be explained by the fact that hippuric acid is a much stronger acid than is monosodium phosphate. The percentage of total acid excreted as ammonium salts was perhaps less on an average, but in no case was the proportion of ammonia so low as in one of the disodium phosphate experiments. The recovery of hippuric acid in the urine was practically complete in every case. The blood showed an increase in alkali reserve in these experiments similar to that shown after the injections of disodium phosphate.

#### DISCUSSION.

These experiments show that the dog can retain the metallic ion from the salts of weak acids as was previously shown for disodium phosphate by Cushny, but the evidence here presented brings out the fact that one sodium ion of disodium phosphate and the sodium ion of sodium hippurate may be completely retained by the fasting dog. At least, this is true for the quantities of salts used in these experiments.

The work on sodium hippurate is especially interesting, inasmuch as hippuric acid is a relatively strong acid. Henderson and Spiro (9) and Henderson (10) have pointed out that not more than 3 per cent of the hippuric acid could exist in the free state, even in those urines which have the highest observed hydrogen ion concentration. In the blood, hippuric acid could exist as the free acid only in negligible amounts. These considerations point to changes in the kidneys of the sort shown by the following equations.



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The phosphate excretion in at least one hippurate experiment was not sufficient to take care of more than the free acid. The ammonia accounted for the remainder of the total acid. As far as the hippurates are concerned, the evidence points to an interchange between  $\text{NH}_4$  ions and Na ions in greater proportion than between H ions and Na ions. It would be interesting to investigate the sodium salts of acids which are stronger than hippuric but weaker than the strong mineral acids, but none which would not be at least partially destroyed in the animal body was available to us.

It would appear that the limit of the power of the kidney to retain metallic ions must be somewhere between hippuric acid and sodium acid sulfate, that the kidney, or the animal body at least, retains the sodium ion of sodium hippurate but not even the second sodium of normal sodium sulfate. The dissociation constant of hippuric acid is given by Henderson and Spiro as  $2.22 \times 10^{-4}$  while that of the second hydrogen of sulfuric acid is about  $3 \times 10^{-2}$  as sodium acid sulfate is about 100 times as strong as hippuric acid. The limit of the power of the kidney to hold back sodium and other metallic ions from salts must be somewhere within the range indicated by these two substances.

The ammonia excretion in both the phosphate and the hippurate series lends support to the hypothesis advanced by Nash and Benedict that the urinary ammonia has its origin in the kidney. It is impossible to suppose that injections of disodium phosphate or sodium hippurate can decrease the pH of the blood or cause an appreciable increase in the amount of available free alkali in the tissues. The older conception of the formation of urinary ammonia assumes that it reacts with acids formed in the tissues and is then excreted by the kidney. Sodium hippurate and disodium phosphate undoubtedly increase the available alkali in the blood and probably also in the tissues, hence if the increase of urinary ammonia observed in this study did not have its origin in the kidney, some new explanation for the formation in the other tissues of the animal body must be found.

In addition to this source of ammonia, it seems worth while to mention again the probability that some of the urinary ammonia is of intestinal origin. If the ammonia, which undoubtedly arises in the alimentary tract from the digestion and putrefaction

of protein, reacts with a strong acid such as hydrochloric to form a salt, it would most likely be absorbed and then excreted by the kidney, in part at least, without further change.

#### SUMMARY.

1. Fasting dogs show an increased excretion by the kidney, of both titratable acid and ammonia after injections of disodium phosphate and of sodium hippurate.

2. The amount of total acid (titratable acid plus ammonia) excreted in the urine was practically equivalent to the salt injected.

3. The urinary ammonia was always increased by the injection of disodium phosphate and of sodium hippurate.

4. Evidence is presented in support of the view of Nash and Benedict that at least a portion of urinary ammonia is formed in the kidney.

5. It is suggested that the kidney can retain sodium from the salts of acids as strong as hippuric, but, obviously, not even one sodium is retained from neutral sodium sulfate or from salts of acids which dissociate as much as the  $\text{HSO}_4$  ion of acid sulfates.

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## THE EFFECT OF CHLOROFORM AND PHOSPHORUS POISONING ON CARBOHYDRATE TOLERANCE.

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In a previous communication (1) we reported the results of a study of the tolerance of normal dogs for glucose, fructose, and galactose. It was pointed out that fructose is less effective and galactose more effective than glucose in producing alimentary hyperglycemia. Dogs show relatively slight variations in their tolerance for fructose. Judging from a number of similar experiments on normal human subjects, the same rule seems to apply. In the case of the dogs, we have never observed an increase of more than 35 mg. of blood sugar following the administration of 3 gm. of fructose per kilo of body weight, and in most instances the increased concentration was not greater than 20 mg. in 100 cc. of blood. On the other hand, no such striking constancy in effects was observed following the ingestion of similar quantities of glucose or galactose.

In the present work we have studied the effect of experimental liver injury on carbohydrate tolerance in the dog. It is well known that normal carbohydrate metabolism is deranged in conditions where the liver is removed or where its function is seriously impaired. Sachs (2) found that the tolerance for levulose was lowered in frogs following liver extirpation. More recently Jacobson (3) has shown that Eck fistula animals have an extremely low tolerance for levulose but that glucose tolerance is only slightly modified in this condition. Roger (4), Baylac (5), and de Haan (6) observed that after the ingestion of sucrose, glycosuria was more frequent in cases of liver involvement than in normal individuals. Strauss (7) attributed the

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lowered tolerance in these cases to a diminished utilization of the levulose arising from the ingested sucrose. He therefore proposed levulose as a test for hepatic function. After the administration of 100 gm. of this sugar, the urine was analyzed for what was presumably levulose; the appearance of a reducing substance being regarded as a positive test. Using this method, later investigators obtained conflicting results. Landsburg (8) and Churchman (9) have shown that the tolerance for levulose as determined by this method is so variable in different individuals that the Strauss test is totally unreliable.

Measurement of carbohydrate tolerance by determining the effect on the blood sugar has yielded more uniform results. Spence and Brett (10) report that in certain cases of toxic jaundice due to arsphenamine, a valuable indication of the efficiency of the liver may be obtained by estimating the changes in the blood sugar concentration which follow the ingestion of levulose. In a number of unpublished experiments, we have found this method useful as an aid in the diagnosis of certain cases of eclampsia, hepatic cirrhosis, and salvarsan jaundice. Very recently Covell (11) employed the levulose tolerance test in determining the degree of hepatic inefficiency in amebic dysentery.

### EXPERIMENTAL.

In the following experiments, normal dogs were used. The tolerance for the monosaccharides was determined as in our previous work (1) using Merck's c.p. glucose and Pfanstiehl's c.p. "special grade" levulose and galactose. The method of Folin and Wu (12) was used in the determination of blood sugar.

Prolonged chloroform anesthesia, as well as the subcutaneous administration of this substance, results in extensive injury to the parenchymatous organs, particularly the liver. In a number of trials we failed to maintain dogs under chloroform anesthesia over prolonged periods. However, in the case of one animal (Dog C-2), three successive administrations of chloroform by the "drop method" resulted several days later in a marked decrease in the tolerance for levulose and dextrose. These data are outlined in Table I. Gross autopsy showed marked fatty degeneration of the liver. The microscopic examination of the tissues of this organ demonstrated protoplasmic and nuclear degenerative changes.

TABLE I.

*Carbohydrate Tolerance Before and After Prolonged Chloroform Anesthesia.*

Dog C-2, male.

Day.	Weight.	Sugar administered.	Blood sugar per 100 cc.					Remarks.
			Initial.	15 min.	45 min.	75 min.	135 min.	
	kg.		mg.	mg	mg	mg	mg	
1	8 7	26.1 gm. levulose.	77	89	87	87	87	Glycosuria.
2	8 7	26.1 " galactose.	81	98	164	212	261	Marked glycosuria.
3	8 7	26.1 " glucose.	99	126	125	122	116	No glycosuria.
4								Chloroform anesthesia for 2 hrs.
5								Anesthesia for 1 hr.
6								" " 1 "
								30 min.
7	8 4	25.2 gm. levulose.	65	97	137	130	122	Glycosuria.
8	8 4	25.2 " galactose.	70	116	190	270	208	Marked glycosuria.
9	8 1	24.3 " glucose.	80	165	209	157	110	Glycosuria.

TABLE II.

*Carbohydrate Tolerance Before and After Chloroform Poisoning.*

Dog C-5, male.

Day.	Weight	Sugar administered.	Blood sugar per 100 cc.					Remarks.
			Initial	15 min.	45 min	75 min	135 min.	
	kg		mg	mg	mg.	mg	mg	
1	6 2	18.6 gm. levulose.	97	112	114	118	93	Glycosuria.
2	6 2	18.6 " galactose.	97	150	220	258	150	"
3	6 2	18.6 " glucose.	105	182	255	254	100	"
4								4 cc. chloroform in olive oil subcutaneously.
6	6 2	18.6 gm. galactose.	90	164	215	276	190	Glycosuria.
7	6 2	18.6 " levulose.	120	165	183	147	122	"
8	6 2	18.6 " glucose.	100	198	260	312	133	"
13	6 0	18.0 " levulose.	92	137	137	113	102	"
18	6 0	18.0 " "	83	116	102	90	83	"



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More severe poisoning was produced in Dog C-5. In this case, 4 cc. of chloroform in olive oil were administered subcutaneously. As indicated in Table II, marked liver inefficiency was observed 3 days later. On the 13th day of the experiment, considerable improvement was noted. Almost complete restoration of function was observed on the 18th day. These results clearly demonstrate the remarkable rapidity with which the liver regains its functional activity following extensive chloroform necrosis. In this regard, our findings accord well with the histological observations made by Davis and Whipple (13). These authors have shown that an injury of one-half of the liver lobule may be repaired in 9 days on a sugar diet. Even if the animal were to receive no food, half repair would occur within this period.

TABLE III

*Levulose Tolerance Before and After Chloroform Poisoning.*

Dog C-18, male.

Day	Weight. kg	Sugar administered	Blood sugar per 100 cc					Remarks
			Initial	15 min	45 min	75 min	135 min	
			mg	mg	mg	mg	mg	
1	7.8	23.4 gm levulose	96	112	128	115	92	2 cc. chloroform.
3	7.0	21.0 " "	121	144	156	125	105	
6	7.0	21.0 " "	125	128	135	136	125	4 cc. chloroform.
7	7.3	21.9 " "	83	135	143	137	108	3 " "
9	7.0	21.0 " "	96	102	150	140	102	
14	7.3	21.9 " "	68	99	119	103	92	

In a third animal, somewhat greater resistance to chloroform was observed. However, even in this case, subcutaneous injections of this substance were followed by a marked decrease in levulose tolerance (Table III).

Phosphorus produces injurious effects upon all the organs of the body, the most marked anatomical injury being observed in the liver where fatty changes usually occur. Phosphorus is especially destructive of the cytoplasm of the liver cells.

Mild phosphorus poisoning was produced in a dog by the administration of 4 mg. of phosphorus in olive oil. On the day following the injection; a decrease in levulose tolerance was

noted, as shown by the data in Table IV. A second dose of 10 mg. of phosphorus ended fatally.

More extensive liver injury was produced in a second dog (No. P-10). As shown by the data outlined in Table V, liver inefficiency, as determined by the levulose tolerance test, was

TABLE IV.

*Levulose Tolerance Before and After Mild Phosphorus Poisoning.*

Dog P-2, male.

Day.	Weight.	Sugar administered.	Blood sugar per 100 cc					Remarks.
			Initial.	15 min	45 min	75 min	135 min	
	kg		mg.	mg	mg	mg	mg	
1	6 5	19 5 gm levulose	77	83	99	92	92	. 4 mg. P. 10 " " Died.
2								
3	6 5	19 5 gm. levulose	101	122	134	126	103	
4								

TABLE V

*Carbohydrate Tolerance Before and After Phosphorus Poisoning.*

Dog P-10, female.

Day.	Weight	Sugar administered	Blood sugar per 100 cc					Remarks.
			Initial.	15 min	45 min	75 min.	135 min	
	kg		mg.	mg	mg	mg.	mg	
1	6 4	19.2 gm. glucose	102	193	226	216	120	10 mg. P.
2	6 4	19.2 " levulose.	84	103	109	107	100	
8								
10	6 0	18.0 gm. glucose.	80	129	140	182	190	
11	6 0	18.0 " levulose.	89	119	131	160	129	Dog died.
12								

noted 3 days after the animal received a subcutaneous injection of 10 mg. of phosphorus in olive oil. The dog died on the following day. Very marked fatty degeneration was observed on autopsy.

The effect on liver function of successive sublethal doses of phosphorus was determined in the case of Dog P-19. This animal received three 5 mg. doses of phosphorus in olive oil, injected subcutaneously. The tolerance for levulose was pro-

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gressively diminished, as indicated by the data outlined in Table VI, the animal dying on the 11th day of the experiment.

We have determined the effect of chronic alcoholism upon levulose tolerance in a single animal. Following an initial fructose test, which was normal, a dog, weighing 12.9 kilos, received daily 50 cc. of alcohol diluted with water. This was continued for a period of 2 weeks. Moderate liver involvement was indicated at the end of this time. The administration of 100 cc. of alcohol on 3 successive days resulted in the death of the dog. Microscopic examination of the liver tissue showed early chronic interstitial hepatitis and slight fatty degeneration. In addition, acute diffuse tubular nephritis was noted.

TABLE VI  
*Levulose Tolerance Before and After Phosphorus Poisoning.*  
Dog P-19, female

Day.	Weight. <i>kg</i>	Sugar administered	Blood sugar per 100 cc					Remarks.
			Initial	15 min	45 min	75 min	135 min	
			<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	
1	13 0	39 gm levulose	96	97	102	99	90	
2								5 mg P.
5	13 0	39 gm levulose	108	110	127	113	108	5 " "
8	12 0	36 " "	68	98	118	151	152	5 " "
10	11 7	35.1 " "	96	115	162	173	153	
11								Dog died.

### DISCUSSION.

A consideration of the results obtained in the preceding experiments leads to the conclusion that the levulose tolerance test, as applied by us, gives reliable information concerning the functional capacity of the liver in dogs. The ingestion of dextrose, on the other hand, produces too variable an effect on the blood sugar concentration to be of similar value as a liver function test. Owing to the low tolerance for galactose normally, any divergence from the normal as a result of liver injury would be too slight to be easily interpreted for purposes of diagnosis.

In the case of animals with severe liver injury, the glycosuria following the ingestion of the monosaccharides is more marked

than in normal animals. It has been pointed out by the writer, previously, that the sugar appearing in the urine of normal dogs after fructose feeding is not levulose, but more likely reducing decomposition products of this substance. In this regard, our findings are in accord with those of Folin and Berglund (14). In conditions of hepatic derangement, however, we have been able to demonstrate alimentary fructosuria (Seliwanoff reaction and preparation of methylphenylfructosazone).

Whipple and Sperry (15) made a careful study of tissue repair in the liver following central necrosis. These authors found that after sufficient chloroform had been administered to cause necrosis of the central two- or three-fifths of every liver lobule, the process of repair would begin at once. It appears that the debris is removed by wandering cells or by cell enzymes. The remaining cells multiply rapidly, the greater part of the deficit being replaced in about 6 days. According to Whipple and Sperry almost complete recovery is effected in 11 days, and after 3 weeks, the liver seems quite normal. Our observations demonstrate that restoration of liver function runs a course parallel to the regeneration of liver tissue as described by Whipple and Sperry. As shown in Table II, 2 days following poisoning with chloroform, there was marked levulose intolerance. 6 days later, there was very noticeable improvement in liver function, with practically a return to normal 14 days after the intoxication.

#### SUMMARY.

A study of carbohydrate tolerance has been made in experimental derangements of the liver due to chloroform and phosphorus poisoning. On the basis of our results, supported by pathological findings, we are led to conclude that the levulose tolerance test, as described, is of great value in measuring the degree of liver involvement in experimental animals. Lowered tolerance for glucose and galactose is likewise associated with severe liver injury. However, glucose cannot be used in testing liver function because other factors may influence the tolerance for this carbohydrate. Similarly, galactose is of relatively little value in this regard owing to the marked individual variations exhibited by different animals in their tolerance for this sugar. As regeneration of liver tissue occurs in chloroform necrosis,

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liver function is rapidly restored. Within 6 to 14 days after poisoning with chloroform, the tolerance for levulose in dogs had returned almost to normal.

The writer is indebted to Professor J. B. Sumner of Cornell University in whose laboratory this work was completed and to Professor H. C. Hartman of the University of Texas who made the microscopic examination of a number of the tissues.

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# THE DETERMINATION OF CHLORIDES IN BLOOD AND TISSUES.

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In the following paper a chloride method is presented in which the sources of error have been reduced by so simplifying the technique that the entire operation is carried out in a single vessel. The proteins are destroyed, and the chloride is precipitated, by heating with concentrated nitric acid in the presence of known amounts of silver nitrate; and the excess silver is titrated with sulfocyanate under the conditions worked out by Whitehorn (1921) for performing the Volhard titration accurately in the presence of the silver chloride precipitate.

In attempting to determine the chloride content of centrifuged blood cells, Van Slyke, Wu, and McLean (1923) encountered difficulties in applying the method devised by Austin and Van Slyke (1920) for whole blood. The formation of the silver precipitate in the protein-free, picric acid filtrate was peculiarly slow, and the above authors were uncertain whether the phenomenon was due to retardation in the precipitation of silver chloride, or to a slow subsequent precipitation of some other silver compound. In either case, it became desirable to improve the method by eliminating the disturbing retardation of the precipitation. In consequence, the oxidation of the organic matter by nitric acid has been resorted to.

The principle of destroying proteins by nitric acid in the presence of silver nitrate, in an open flask (performing, so to say, an open Carius determination) appears to have been first used by von Korányi (1897). In order to avoid the necessity of precipitating the protein in urine before titrating the chloride, he added concentrated nitric acid with the standard silver nitrate and heated till the proteins were dissolved, adding permanganate to assist in clearing the solution. He applied the method only to urine, and gave no figures to show whether the process involved loss of chlorine or gave accurate results with standard chloride solutions.

Rusznýák and Kellner (1922) have recently recommended ashing blood and tissues with nitric acid *before* the silver nitrate is added. They likewise give no control determinations on material of known chloride content. We find that a large percentage of the chlorine is lost by their procedure.

Greenwald and Gross (1922) have deproteinized blood for chloride determination by heating it with nitric acid in the presence of excess silver nitrate. They presented no data on known chloride solutions demonstrating quantitative recovery, but the constancy of the results with blood, and the approximation to results by other methods, indicated the probability that volatilization of chlorine was prevented by the silver nitrate.

In the present paper we have tested the open Carius method by analysis of standard aqueous KCl solutions, and of known solutions prepared by addition of weighed amounts of KCl to blood and blood cells which had been dialyzed until chloride-free. We have also confirmed the accuracy of the Volhard titration of the excess silver, without removal of the AgCl, under the conditions of nitric acid and ferric alum concentration adopted by Whitehorn.

#### *Macro Method.*

*Procedure.*—To 3 or 5 cc. (or grams) of *serum* or *blood* in a 100 cc. Erlenmeyer flask of Pyrex glass are added 10 or 15 cc., respectively, of 0.05 N AgNO<sub>3</sub>, prepared in concentrated nitric acid (of 1.4 specific gravity). The flask is covered with a watch-glass and heated on a water bath till the solution above the AgCl precipitate is clear and light yellow in color. For this purpose 1 to 2 hours suffice for serum, while whole blood requires about 12 hours (we usually leave it overnight). For each cc. of the nitric acid solution used 2 cc. of water are then added (20 or 30 cc.). Powdered ferric alum (1 or 1.5 gm. measured approximately from a spoon or tube) is added, the solution is cooled to room temperature, and the excess silver is titrated with 0.05 N sulfocyanate. An empirical correction of 0.04 cc. is subtracted from the sulfocyanate used, as under the conditions of the titration this excess is found necessary to give a decided end-point.

For *tissues* the procedure is the same, except that their smaller chloride content makes it desirable to digest with nitric acid containing 0.02 N instead of 0.05 N AgNO<sub>3</sub>. With tissues it is desirable also to add 1 or 2 drops of octyl alcohol to prevent foaming at the beginning of digestion.

*Calculation.*—Milli-equivalents of Cl per liter or kilo =

$$\frac{50 (\text{cc } 0.05 \text{ N Ag} - \text{cc. } 0.05 \text{ N CNS})}{\text{cc. or gm. sample}}$$

Cl calculated as gm. of NaCl per liter or kilo =  $0.0585 \times$   
m.-Eq. =

$$\frac{2.925 (\text{cc. } 0.05 \text{ N Ag} - \text{cc. } 0.05 \text{ N CNS})}{\text{cc. or gm. sample}}$$

Control titrations are made to standardize the thiocyanate. 15 cc. of the 0.05 N  $\text{AgNO}_3$  plus 35 cc. of water plus 1.5 gm. of ferric alum are titrated directly against the thiocyanate. If the volume required of the latter varies from 15.00 cc., the actual "cc. 0.05 N CNS" is obtained by multiplying the cc. of thiocyanate used by the factor  $\frac{15.00}{\text{cc. CNS used in control}}$ .

#### *Micro Method.*

*Procedure.*—1 cc. of blood, or 1 to 1.5 gm. of tissue, is placed in a 100 cc. test-tube of Pyrex glass (we use the tubes employed in the Van Slyke-Cullen (1916) urea determination), and 3 cc. of the concentrated nitric acid containing  $\text{AgNO}_3$  in 0.05 N concentration are added. The tube is covered with a watch-glass and the mixture is digested as described above. Then 6 cc. of a 5 per cent solution of ferric alum in water are added, the solution is cooled, and the excess silver is titrated with 0.02 N sulfocyanate. A correction of 0.04 cc. is subtracted from the 0.02 N sulfocyanate used.

*Calculation.*—m.-Eq. of Cl per liter or kilo =

$$\frac{20 (7.50 - \text{cc. } 0.02 \text{ N CNS})}{\text{cc. or gm. sample}}$$

Gm. NaCl per liter or kilo =

$$\frac{1.170 (7.50 - \text{cc. } 0.02 \text{ N CNS})}{\text{cc. or gm. sample}}$$

Control titrations are made in which 3 cc. of the 0.05 N  $\text{AgNO}_3$  +  $\text{HNO}_3$  solution plus 6 cc. of ferric alum solution are titrated directly against the 0.02 N sulfocyanate. If the amount of the latter varies from 7.50 cc., the "cc. CNS" figure in the formula is to be multiplied by the factor  $\frac{7.50}{\text{cc. CNS used in control}}$ .



*Standard Solutions.*

The 0.05 N  $\text{AgNO}_3$  solution may be made by dissolving the calculated weight of fused  $\text{AgNO}_3$  ( $0.05 \times 169.9 = 8.495$  gm. per liter) in a minimum amount of water and making up to volume with nitric acid of 1.4 specific gravity. The 0.02 N solution is made by dilution of the 0.05 N with nitric acid.

For accurate standardization it is advisable to prepare one solution by dissolving 5.394 gm. of pure silver in nitric acid, and making up to 1 liter by addition of more acid.

The ammonium, sodium, or potassium sulfocyanate is standardized as usual against the silver, as described in connection with the procedures, and the standardization is repeated at intervals of at most 2 weeks to guard against change.

## EXPERIMENTAL.

*Analyses of Standard Solutions.*

0.25 N KCl solution was prepared by weight from recrystallized KCl (18.64 gm. diluted to 1 liter). It was standardized by diluting 15 cc. samples to 75 cc., acidifying with  $\text{HNO}_3$ , and precipitating with 20 cc. of 5 per cent silver nitrate solution. The latter was added drop by drop with constant stirring. The precipitate was finally coagulated by boiling, and allowed to stand overnight before filtering. The precipitate was collected on a Gooch crucible, and dried overnight at  $110^\circ\text{C}$ . The weights of the precipitates obtained in duplicate determinations were 0.5396 and 0.5396 gm. as compared with 0.5375 gm. theoretical.

To test the accuracy of our titration method the following solutions were prepared from the 0.25 N KCl above described.

*Water Solution of KCl.*—To 25 cc. of water 10 cc. of 0.25 N KCl were added. Neglecting the slight volume change involved in the dilution, the concentration of the solution is  $\frac{10}{35} \times 0.25 = 0.07143$  N.

*Blood Solution of KCl.*—Horse blood was laked with saponin and dialyzed under pressure in closed collodion sacs against repeated changes of distilled water for 4 days. At the end of this time portions of the solution were found to be chloride-free when the proteins were precipitated with saturated picric acid solution, and the filtrate was tested for chloride. To 25 cc.

portions of the dialyzed blood thus prepared 10 cc. portions of the 0.25 N KCl were added.

Of the above water and blood solutions 5 cc. portions were digested overnight and analyzed as described above under "Macro method," and 1 cc. portions were used for micro determinations.

The amount of excess of sulfocyanate required to give the end-point under the conditions of the titration was ascertained by digesting blood with nitric acid without silver nitrate. To the yellow solution was added the thoroughly washed AgCl-

TABLE I.

*Analyses of Known Solutions of KCl in Water and Dialyzed Blood Macro Method. 5 Cc. Samples.*

Solution	0.05 N AgNO <sub>3</sub>	0.05 N NH <sub>4</sub> CNS	0.05 N Ag precipitated by Cl		Cl per liter.	
			From un- corrected titration.	From titration corrected for 0.04 cc excess CNS required for end-point	From corrected titration figures	Added.
	cc	cc	cc	cc	m.-Eq	m.-Eq.
Water solu- tion.	15 00	7 90	7 10	7 14	71 4	71.43
	15 00	7 90	7 10	7 14	71 4	71.43
	15 00	7 90	7 10	7 14	71 4	71 43
Blood solu- tion.	15 00	7 94	7 06	7 10	71 0	71 43
	15 00	7 93	7 07	7 11	71 1	71 43
	15 00	7 93	7 07	7 11	71 1	71 43

AgCNS precipitate obtained from another analysis. Water and ferric alum as usual were added, and standard sulfocyanate was run in from a small burette until the end-point was definite. The amount required was 0.05 cc. of 0.05 N sulfocyanate in the macro method, and the same volume of 0.02 N sulfocyanate in the micro method.

The results of the analyses by the macro method are given in Table I, those by the micro method in Table II.

Comparative routine plasma analyses by the micro method and the Austin-Van Slyke (1920) method, respectively, have given nearly identical results.

We have tried as a further check on our titration to perform parallel gravimetric analyses, similar to the gravimetric analyses of Greenwald and Gross (1922), weighing both the AgCl precipitated by the Cl in the sample and that obtained by adding excess HCl to the filtrate from the first precipitate. Owing apparently to occlusion of unoxidized fat, however, the total AgCl obtained was equivalent to about 110 per cent of the Ag added, the occluded matter being chiefly in the first AgCl precipitate.

TABLE II

*Analyses of Known Solutions of KCl in Dialyzed Blood. Micro Method. 1 Cc Samples.*

AgNO <sub>3</sub>		0.02 N NH <sub>4</sub> CNS		Difference	Cl per liter	
0.05 N used	0.02 N equivalent	Uncorrected	Corrected for 0.04 cc excess required for end-point	0.02 N AgNO <sub>3</sub> - 0.02 N NH <sub>4</sub> CNS	Found	Added
cc	cc.	cc	cc	cc	m-Eq	m-Eq
3.00	7.50	3.95	3.91	3.59	71.8	71.4
3.00	7.50	3.95	3.91	3.59	71.8	71.4
3.00	7.50	3.96	3.92	3.58	71.6	71.4
3.00	7.50	3.98	3.94	3.56	71.2	71.4

### *Chloride Determinations in Tissues.*

A rabbit was dissected and portions of brain, triceps muscle, and liver were analyzed as described under the "Macro method" for whole blood. At the beginning of digestion 1 or 2 drops of octyl alcohol were added to prevent foaming. The results are given in Table III. It will be seen that when separate pieces of tissue were taken the results from the same tissue varied considerably, as might be expected. When the liver was hashed into a uniform suspension, however, the results were about as uniform as those obtained with blood. The accuracy of the method with tissues has not been demonstrated as completely as with blood, by removing the tissue chlorides and restoring known amounts. Yet the similarity of the behavior of the tissues with that of blood, and the constancy of the results obtained with uniform liver suspensions, appear to leave little doubt as to the reliability of the method for tissues.

TABLE III.  
*Tissue Chlorides.*

Tissue.	Chloride per kilo.
	mm.
Brain (separate sections).....	3 88
	3 77
Triceps muscle (separate sections).. . . . .	1 32
	1 12
	1 32
	1 14
Liver (separate sections). . . . .	2 91
	3 11
	3.12
	3 15
	2 77
Liver (uniform mixture).. . . . .	2 82
	2 81
	2 87
	2 80
	2 83

The experimental work in connection with the above paper has been performed entirely by Mr. Julius Sendroy, Jr.

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# THE INFLUENCE OF IRRIGATION WATER ON THE COMPOSITION OF GRAINS AND THE RELATIONSHIP TO NUTRITION.

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The irrigation water applied to a soil not only governs the quantity of grain grown on a unit area, but it also governs the quality. The value of grain produced on a unit area is governed by composition as well as quantity. The quantity and quality of ash found within various grains is of interest to the consumer and producer—to the consumer because it governs in a measure the nutritive value of the product, and to the producer because it represents the fertility which is being taken from the soil. For these reasons, in the future more consideration will be given to this phase of food composition than has been the case in the past.

The great volume of work which is being carried on at the Utah Experiment Station on the influence of water on the yield of plants offers excellent opportunities for a study of the influence of this factor on the composition of grains. Therefore, the results reported in this paper represent total ash, nitrogen, phosphorus, potassium, calcium, and magnesium content of wheat, oats, and barley which were grown on the same soil having a variation in moisture content. The analyses were all made in duplicate on composite samples composed of from three to five yearly yields and reported on the dry basis of the grains. The results as reported in each table are averages of from six to ten separate analyses made on grain from three to five plats receiving like quantities of irrigation. Each of these samples is further made up of a composite of from three to five yearly yields. The yield of grain and treatment are those given by Harris (1) and Harris and Pittman (2). The analyses were made according to the Official Methods (3).

The grain was grown on the Greenville Experimental Farm—a very productive calcareous loam of sedimentary origin. The surface acre-foot contained 4,904 pounds of total nitrogen, 2,700 pounds of total phosphorus, 60,560 pounds of total potassium, 434,365 pounds of acid-soluble calcium, and 132,463 pounds per acre of acid-soluble magnesium. It is probable that the calcium and magnesium occur in the soil mainly as the double salt form  $\text{CaMg}(\text{CO}_3)_2$ , and, therefore, magnesium carbonate itself is present in small quantities and hence not harmful. 0.57 and 0.92 per cent of the soil are soluble in hydrochloric acid of specific gravity of 1.115. The total nitrogen content of grain grown on

TABLE I.

*Percentages and Pounds per Acre of Total Nitrogen Found in Grain of Wheat, Oats, and Barley Grown with Varying Quantities of Irrigation Water.*

Treatment.	Nitrogen					
	Wheat		Oats		Barley	
	per cent	lbs per acre	per cent	lbs per acre	per cent	lbs per acre
No irrigation water	2 392	53 82	2 753	40 17	2 057	25 96
5 in irrigation water	2 161	44 88	2 483	41 54	2 029	27 95
10 “ “ “	2 183	47 14	2 438	48 51	2 075	33 47
15 “ “ “	1 995	42 38	2 332	54 87	1 813	32 04
20 “ “ “	1 976	37 75	2 296	52 03	1 807	33 14
35 “ “ “	2 014	45 12	2 140	55 26	1 773	34 72
45 “ “ “			1 970	47 72		
52 5 “ “ “					1 736	32 26
67 5 “ “ “	2 057	44 70				

this soil with varying quantities of irrigation water is given in Table I.

The nitrogen content of the wheat decreases as the quantity of irrigation water increases. This is pronounced with small applications, and where the wheat was grown with large applications the nitrogen reaches what may be termed an irreducible minimum. There is 0.416 per cent less nitrogen in the grain grown on land receiving 20 inches of irrigation water over that grown on non-irrigated soil, and the total quantity of nitrogen contained in the non-irrigated grain is 43 per cent greater. Although the results are very irregular the tendency is for the total pounds per acre in the various grains to increase up to a certain maximum as the

irrigation water applied increases, after which there is a decline. This is very pronounced in the oats and barley. It is probably correlated with the quantity of nitrates produced in this soil, as it has been shown elsewhere (4) on this same soil that the nitrate production is influenced in the reverse order. However, the quantity which remains at the surface and can be utilized by the growing plant is in the same order as the increased nitrogen in the plant.

The oats show a very regular decrease in the percentage of nitrogen as the quantity of irrigation water increases. The oats raised on non-irrigated soil contained 39 per cent more nitrogen than those grown on soil receiving 45 inches of water. The barley follows the same general law.

This decrease in nitrogen with increased irrigation is due to two factors: (a) The irrigated grain due to its greater demand for nitrogen is not able to obtain sufficient from the limited supply of the soil to build a high protein grain. This is borne out by the findings of Gericke (5) that a high protein grain can be produced by keeping a ready supply of nitrates within reach of the growing plant. (b) The application of large quantities of irrigation waters during the growing season washes the nitrates beyond the feeding powers of the roots. This is borne out by these results, for it is found that where the water was applied in small frequent applications the protein of the grain was high as compared to grain grown on land receiving the same quantities of water but in large applications. The small frequent applications bring the soil up to an optimum (6) for rapid nitrification, but do not carry the nitrates beyond the feeding area of the roots; hence, the higher protein grain. Applications of large quantities of water to the more mature plant have greater effects in reducing nitrogen content than have applications earlier in the growing period because it is during this latter period that a full supply is necessary to build the high protein grain. Excess water at this period carries it beyond the feeding area of the plants.

The ash content of these same grains is given in Table II.

The ash of the grain of wheat, oats, and barley increases with an increase of irrigation water. The ash of the wheat grown on land receiving 67.5 inches of irrigation water was slightly less than that grown with 35 inches. Probably the maximum ash



content is reached when wheat is grown with somewhat less than 67.5 inches of irrigation water. Probably the ash content of wheat would not increase above this with increased water. These results are significant both to the producer and consumer, for we find that the producer sells wheat with 46 per cent more ash, oats with 31 per cent more, and barley with 36 per cent more when grown with large quantities of water than when grown with small quantities. This would be 22 pounds for a 50 bushel crop of wheat, 33 pounds for a 100 bushel crop of oats, and 39 pounds for a 75 bushel crop of barley. This excess all comes from the soil and hence would be produced at a higher cost per unit than would grains produced with less water in which the ash is low.

TABLE II

*Percentages and Pounds per Acre of Total Ash Found in Grain of Wheat, Oats, and Barley Grown with Varying Quantities of Irrigation Water.*

Treatment	Ash.					
	Wheat		Oats.		Barley	
	per cent	lbs per acre	per cent	lbs per acre	per cent	lbs per acre
No irrigation water .	1 561	35 12	3 344	48 79	2 366	29 86
5 in. irrigation water	1 561	34 17	3 524	65 67	2 329	32 03
10 " " "	1 568	35 90	3 597	74 27	2 332	38 07
15 " " "	1 711	36 38	3 664	87 92	2 723	48 51
20 " " "	2 015	38 45	3 606	81 81	2 810	51 54
35 " " "	2 284	51 13	4 288	110 70	2 978	58 31
45 " " "			4 390	106 30		
52 5 " " "					3 228	59 98
67 5 " " "	2.194	47.13				

For the feeding of farm animals in which the production of bone is considered the irrigated grain would be superior. Whether or not watered grain would be more valuable in the bread of man would depend upon whether the milling process were leaving the same quantities of ash in the watered grain and whether the excess of ash were more valuable than the excess of protein in the non-irrigated grain. Individuals in need of more ash in the diet could well turn to the irrigated grains in preference to the non-irrigated, although by so doing they would be getting less protein, and conversely where it is desired to restrict the mineral intake of an individual the non-irrigated grain should be used. Although

larger application of irrigation water in many cases reduced the yield, yet in all but one case the total ash removed in the grain increased with the water applied.

The phosphorus content of the various grains is given in Table III.

Wheat, oats, and barley all show a gradual progressive increase in phosphorus as the quantity of irrigation water used in their production increases up to 35 inches yearly. Above this there is a decrease. The increase in the case of wheat amounts to 55 per cent, oats 35 per cent, and barley 30 per cent. This means that it would require for a 50 bushel crop of wheat 4.9 pounds, a 100

TABLE III.

*Percentages and Pounds per Acre of Total Phosphorus Found in the Grain of Wheat, Oats, and Barley Grown with Varying Quantities of Irrigation Water*

Treatment	Phosphorus					
	Wheat		Oats		Barley.	
	per cent	lbs per acre	per cent	lbs per acre	per cent	lbs per acre
No irrigation water	0.2953	6.64	0.2793	4.07	0.3090	3.90
5 in. irrigation water	0.3011	6.38	0.2870	5.84	0.3024	4.30
10 " " "	0.3059	6.38	0.3131	6.23	0.3001	4.70
15 " " "	0.3233	6.87	0.3182	7.51	0.3158	5.59
20 " " "	0.3710	7.08	0.3400	7.71	0.3349	6.14
35 " " "	0.4578	10.24	0.3782	9.77	0.4023	7.88
45 " " "			0.3690	8.94		
52 5 " " "					0.3752	6.97
67 5 " " "	0.4245	9.12				

bushel crop of oats 2.9 pounds, and a 75 bushel crop of barley 4.2 pounds more of phosphorus if grown with large quantities of water than if grown with small quantities. Conversely, it means that the user of grains obtains these greater quantities of phosphorus if he purchases grains grown with these quantities of water. However, this should not be taken to mean that the increase of phosphorus has the same nutritive value as the minimum found in the grains grown with small quantities of water. The nitrogen content varies inversely with the water applied, and the phosphorus content of the grains varies directly with the water. Hence, the increased phosphorus content is probably inorganic

and not phospho- and nucleoproteins which would be of less value to the animal.

However, this extra phosphorus would not be without value, for animals kept on a grain diet grown on irrigated grain would probably find the extra quantities sufficient to build strong bones, and if fed to milk cows it would probably modify the calcium and phosphorus content of the milk (7). This in turn would modify the nutritive value and might modify the digestibility of the milk. Whether this increase would more than offset the value of the extra protein in the wheat remains to be determined. It does indicate, however, that the feeder may often use a calculated mixed ration of the irrigated and non-irrigated grains, depending upon whether more ash or more protein is needed in the food.

The total quantity taken from the soil also varies with the quantity of water used in its production up to 35 inches yearly. Above this there is a decrease. Very likely the increase in phosphorus content is associated with an increase in the available phosphorus of the soil, for as the water content of the soil increases up to a certain level there is a proportional increase of the soil microflora (6) which would increase the quantity of acids. These in turn liberate phosphorus from its difficultly soluble form. When the optimum moisture (6) is exceeded there is a proportional decrease in bacterial acids. This would result in less available phosphorus which manifests itself in a grain with a lower phosphorus content. Wheat is modified in phosphorus content to a greater extent than either of the other grains. It also carries greater quantities of total phosphorus from the soil under all the irrigation treatments than does either oats or barley.

In Table IV is given the potassium content of these same grains.

The percentages of potassium in the wheat increased progressively with the water applied so that by the time it was receiving 35 inches of irrigation water it was carrying 35 per cent more potassium than was the wheat grown with no irrigation water. The ratio of phosphorus to potassium where grown with no irrigation water is 1:1.34. This ratio becomes wider as the water used increases to 15 inches where the ratio is 1:1.53. Above this it grows narrower, and wheat grown with 67.5 inches

of water has a phosphorus-potassium ratio of 1:1.26. Therefore, water has a greater influence on the phosphorus content of wheat than it has on its potassium content.

The oats increase in potassium content as the water applied increases. Those grown with 45 inches of water contain 31 per cent more potassium than those grown without irrigation water. The ratio of phosphorus to potassium in the oats is wider than in the wheat, and the variation in this ratio is in the same order as in the wheat.

The barley increases in potassium as the irrigation water increases up to 20 inches. Above this there is a decrease. Water

TABLE IV.

*Percentages and Pounds per Acre of Total Potassium Found in the Grain of Wheat, Oats, and Barley Grown with Varying Quantities of Irrigation Water.*

Treatment	Potassium					
	Wheat		Oats		Barley	
	per cent	lbs per acre	per cent	lbs per acre	per cent	lbs per acre
No irrigation water	0 3965	8 920	0 4176	6 090	0 3886	4 90
5 in irrigation water	0 4137	8 970	0 4833	8 120	0 4007	5 47
10 " " "	0 4395	9 490	0 4827	9 680	0 4467	7 22
15 " " "	0 4915	10 470	0 4736	11 170	0 4773	7 24
20 " " "	0 4902	9 350	0 4741	10 740	0 5462	10 02
35 " " "	0 5340	11 950	0 5212	13 460	0 5159	10 10
45 " " "			0 5461	13 23		
52 5 " " "					0 4443	8 26
67 5 " " "	0 5351	11 50				

increases the potassium in the wheat by 35 per cent, which is less than the increase in the phosphorus content of the wheat. The oats increase 31 per cent and the barley 14 per cent due to irrigation water. The ratio of phosphorus to potassium in the barley is narrower than in the wheat and oats, but follows the same order of variation as it does in these grains, thus indicating that the same law is operating in all three grains in the uptake of phosphorus and potassium and is probably correlated with the available constituents of the soil. Both are rendered more soluble by an increased bacterial activity due to increased water content of the soil.

The calcium content of the three grains is given in Table V.

The calcium content of the wheat increases as the irrigation water increases. This increase is greatest with small applications and is approximately constant for each increase of 5 inches of water up to 20 inches. Wheat raised with 67.5 inches of irrigation water contains 2.55 times as much calcium as does wheat grown with no irrigation water. Such differences as these must have significance in human nutrition, for where the whole grain is used the individual would be getting much greater quantities of calcium. Whether this difference persists—which it probably does—in the milled grains remains to be answered.

TABLE V.

*Percentages and Pounds per Acre of Total Calcium Found in the Grain of Wheat, Oats, and Barley Grown with Varying Quantities of Irrigation Water.*

Treatment	Calcium					
	Wheat		Oats		Barley	
	per cent	lbs per acre	per cent	lbs per acre	per cent	lbs per acre
No irrigation water	0 1027	2 310	0 1464	2 140	0 1066	2 80
5 in irrigation water	0 1072	2 020	0 1480	2 880	0 1029	2 95
10 " " "	0 1221	2 630	0 1679	3 540	0 1034	3 28
15 " " "	0 1651	3 530	0 1668	3 600	0 1069	4 19
20 " " "	0 1951	3 780	0 1783	4 040	0 1020	3 89
35 " " "	0 2106	4 720	0 1598	4 130	0 1448	5 90
45 " " "			0 1356	3 28		
52 5 " " "					0 1502	5 81
67 5 " " "	0 2625	5 64				

There is an increased calcium content in the oats until the irrigation water applied reaches 20 inches. With 35 inches and above there is a decrease. The barley shows a persistent gain in calcium with increased irrigation water. That grown with 52.5 inches contains 1.41 times as much as that grown without irrigation water. The ratio of the calcium to the phosphorus is greatest in the barley and least in the oats. There is a marked uniformity between the increase in calcium and of phosphorus of the various grains where grown with increased irrigation water thus indicating that the increase of these elements is deposited as inorganic.

The magnesium content of the various grains is given in Table VI.

The magnesium content of all three grains increases as the irrigation water used in their production increases. Wheat showed an increase of 32 per cent, oats 65 per cent, and barley 9 per cent. The ratio of magnesium to phosphorus is quite uniform for the various grains grown with different quantities of water. The magnesium-phosphorus ratio in all the samples for wheat is 1:1.91, for oats 1:1.87, and for barley 1:1.84. This is considerably narrower than is the calcium-phosphorus ratio which is as follows: wheat, 1:2.19; oats, 1:2.09; and barley, 1:2.88.

TABLE VI.

*Percentages and Pounds per Acre of Total Magnesium Found in the Grain of Wheat, Oats, and Barley Grown with Varying Quantities of Irrigation Water.*

Treatment	Magnesium					
	Wheat		Oats		Barley	
	per cent	lbs per acre	per cent	lbs per acre	per cent	lbs per acre
No irrigation water	0 1698	3 82	0 1319	1 92	0 1794	2 26
5 in. irrigation water	0 1708	3 54	0 1639	2 77	0 1770	2 42
10 " " "	0 1718	3 65	0 1742	3 47	0 1776	2 86
15 " " "	0 1724	3 23	0 1743	3 91	0 1863	2 95
20 " " "	0 1978	3 77	0 1721	3 90	0 1950	3 58
35 " " "	0 2070	4 64	0 1949	5 03	0 1709	3 35
45 " " "			0 2181	5 28		
52 5 " " "					0 1852	3 44
67 5 " " "	0 2236	4 80				

The calcium-magnesium ratio grows narrower in the wheat and wider in the oats and barley as the irrigation water used in their production increases. It is narrow in the case of oats (1:1.13), wider in the wheat (1:1.17), and still wider in barley (1:1.60).

Considering the extent to which irrigation water has modified the mineral elements in these grains it is easy to see how a ration of irrigated cereals would carry sufficient calcium and phosphorus if fed to swine to produce strong normal bones, which is not the case with corn alone. Moreover, it is possible that the variations sometimes obtained by different feeders may be correlated with this large variation in ash content of grains grown under different conditions of irrigation.

Moreover, there may be cases where human individuals are living on restricted diets in which the greater quantities of minerals contained in the irrigated grains may be sufficient to prevent nutritional disorders that may occur where the dry farm grains are used.

It is certain that the results are significant to the dietitian who is considering the feeding of the individual with kidney disorders. If fed on the irrigated grains the individual may be getting from 26 to 46 per cent more ash than if fed on the dry farm grains. Whether the dry farm grains are more nutritional to the normal individual would depend upon the remainder of his diet. If it be low in protein the non-irrigated grains would be better, whereas if low in ash the irrigated grains would be indicated.

These results strongly point to the fallacy of overirrigation from the standpoint of soil fertility for it is depleting the soil in two ways: (a) It washes out the soluble nitrogen which is a limiting factor in soil fertility in the arid region and thus produces a low protein grain and (b) it causes the grains to take up larger quantities of potassium and phosphorus than they otherwise would, thus unnecessarily depleting the soil and as a result the farmer receives less per unit for each of these essential elements which are sold from his farm.

#### SUMMARY.

Wheat, oats, and barley were found to decrease in nitrogen as the irrigation water used in their growth increased. This decrease for wheat was 21 per cent, oats 40 per cent, and barley 19 per cent.

The quantity of ash in the grain increased progressively as the irrigation water used in its production increased. This increase was for wheat 46 per cent, oats 31 per cent, and barley 36 per cent.

The percentage of phosphorus in these grains increased as the water applied increased. The increase was 55 per cent for wheat, 35 per cent for oats, and 30 per cent for barley.

A similar increase occurred in the case of potassium which was 35 per cent for wheat, 31 per cent for oats, and 14 per cent for barley.

The calcium content increased due to water 155 per cent for wheat, 22 per cent for oats, and 41 per cent for barley.

The magnesium content increased due to irrigation water 32 per cent for wheat, 65 per cent for oats, and 9 per cent for barley.

The ratios of calcium and magnesium to phosphorus indicate that the increase in phosphorus is mainly inorganic.

The authors wish to acknowledge their indebtedness to Professor Pittman for placing at their disposal samples of the grains and for furnishing data on yields and treatment.

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## BODY FLUID OF THE HONEY BEE LARVA.

### I. OSMOTIC PRESSURE, SPECIFIC GRAVITY, pH, O<sub>2</sub> CAPACITY, CO<sub>2</sub> CAPACITY, AND BUFFER VALUE, AND THEIR CHANGES WITH LARVAL ACTIVITY AND METAMORPHOSIS.

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Recent work on the blood gases and pH has dealt almost exclusively with mammalian blood, which is a highly specialized type of body fluid, in that its chief oxygen carrier is confined to cells, and the O<sub>2</sub> and CO<sub>2</sub> exchanges affect each other reciprocally and indirectly through the passage of HCl across the cell boundary. The bee larval body fluid has no blood pigment or oxygen carrier capable of being reduced by ferricyanide, has no blood cells comparable to red corpuscles, is not confined to vessels, and does not clot, but has a fair buffer value and is rich in proteins, coagulating readily in weak acid and even in distilled water. It has normally a more acid reaction than mammalian blood, and a CO<sub>2</sub> tension around 35 to 60 mm. of Hg, both varying somewhat with conditions within the hive and with activity. It comes in direct contact with the tissues, which bound the large hemal space, is circulated by a dorsal heart, and comprises 25 to 30 per cent of the body weight.

Aside from the general interest of a comparison between a more simple body fluid such as this is with mammalian blood, the present investigation was undertaken as a further step in the study of the metabolism of the insect food storage mechanism. The larval fat body makes up in the bee so large a proportion of the total larval mass (65 per cent by weight), that up to the transformation to pupa the larval metabolism is predominantly the basal metabolism of this tissue plus its growth metabolism; that is, the metabolism of a single tissue is here virtually

isolated under natural conditions. The morphology of this tissue and the cytology of its cell elements have been dealt with previously (Bishop, 1922) and also its rôle in the insect larval cycle (Bishop, 1923). For the purpose of this paper it is sufficient to remark that, the larva being totally inactive, except during spinning, practically all the nutriment for pupal development is stored in the fat body cells and in the blood, the larval organs being much reduced; development of pupal tissues does not commence until after the larva has stopped feeding, whereupon the cells of the fat body break down to furnish nutriment for the growth of the mature insect, and in this breakdown elements from the nuclei invade the cytoplasm of the cells and apparently aid in bringing about the reduction of the cell constituents to substances soluble in the blood and available for use in tissue growth. The blood may thus be looked upon as the nutrient medium for the growth of the fat body tissue during larval life, and as the immediate destination of its histolytic debris during disintegration of the larval tissue. Since histolysis of tissue results in acid production, the buffer action of the blood is significant aside from its particular  $\text{CO}_2$  buffering action. Finally, during spinning of the cocoon at the beginning of pupation, the larva offers a case of muscular activity in a fasting animal, since the contents of the stomach are regurgitated.

A typical cross-section of a larva is reproduced in a previous paper (Fig. 1 (Bishop, 1922)).

#### *Specific Gravity.*

The specific gravity of the blood was determined in a small bulb, filling to a line on the capillary neck, and weighing full of water and of blood at room temperature. It was assumed that blood would have virtually the same coefficient of expansion as water within the accuracy required, and no temperature correction was made. The mean of three readings, 1.044, 1.048, and 1.043 gm., was 1.045 gm. per cc. for worker bee larval blood.

These determinations were used for measuring samples of blood in later experiments, the pipette being weighed full, and after delivery.

Weight gm.	volume cc.
Density gm. per cc.	

*Osmotic Pressure.*

Osmotic pressure was determined by the freezing point method with a thermopile, one end of which was dipped into freezing water, the other into the freezing blood (Fig. 1). Difference of potential was read on a potentiometer. The thermopile consists of ten junctions, five in each solution, of constantan iron wire, sealed with wax into a glass U-shaped tube (*u*). The thermal junctions at either end *p* are arranged around the periph-

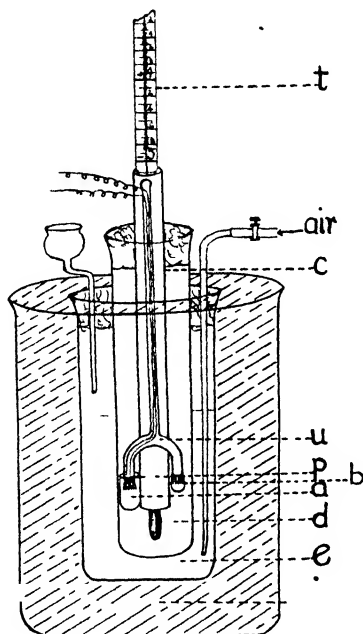


FIG. 1 Freezing point apparatus  $\frac{1}{2}$  size For explanation, see text.

ery of the bore of the tubing, so that the solution is in contact with all of them on all sides. The points are insulated by dipping into a hot beeswax-paraffin mixture. The water chamber *a* holds 15 cc., the blood chamber *b* 0.3 cc.; both consist of short glass tubes fastened to a paper cylinder *c*, sliding up and down on a freezing point thermometer *t*. The whole fits loosely into a large test-tube *d*, dipping into a well insulated ether bath through which air is bubbled, regulated by a screw pinch-cock.

The thermopile can be raised or lowered, by a glass rod attached, and the end of the glass tube enclosing it fits the blood chamber snugly as a cover. Mercury in the bottom of the test-tube chamber hastens the cooling.

In operation, the solutions are cooled in the mercury bath to  $0.5^{\circ}$  below freezing point of the blood, then raised above the mercury, the thermopile raised, and the surface of the water touched with an ice crystal on the end of a glass rod. Freezing is indicated by throw of the galvanometer, whereupon the potential is adjusted to a null-point. The surface of the blood is then touched, when the galvanometer throws the other way, and the thermopile is let down to cover the blood again, and the potential difference is read. Solutions are agitated by slightly raising and lowering the thermopile. The least difference of potential gives the value of  $\Delta$ . The thermopile was checked and calibrated against known salt solutions, and a similar thermopile with longer terminals was read in two water baths. Temperature can be read accurately to  $\pm 0.002^{\circ}$  on 0.2 cc. of blood.

The osmotic pressure in terms of freezing point lowering of water, for larval stages, unsealed, lies at  $-0.86$  to  $-0.87^{\circ}\text{C}$ . This seems to increase (lower freezing point) when the larvæ are spinning, though hardly enough to be outside the variation in the different readings. During pupation the osmotic pressure decreases, reaching the level  $-0.80$  in 1 or 2 days, and  $-0.75$  the 3rd or 4th day of pupation. The cause of this cannot be determined without further chemical study of the blood. Straus (1911) finds a marked decrease in glycogen and fat as pupation goes on, but little change in dry weight percentage. The larvæ at this later stage show much histolytic debris in the blood from fat body disintegration, changes in connection with which might decrease the osmotic pressure. It is not accurately known what is being taken out of the blood by the growing tissues, nor what chemical changes disintegration of larval tissues involves. Further investigation of this phase of the work is in progress.

#### *Blood pH.*

Material was obtained from the larvæ without removing from the comb, by tearing down the side of the cell and pricking, when a drop oozed from the larva. Two needles soldered to the points

of forceps were thrust through the skin and sprung apart. Blood was taken up under oil in a small glass bulb with rubber bulb attached. A screw clamp on the latter was screwed shut, then opened gradually as blood was taken up, and a capillary point allowed a slight negative pressure to be maintained in the pipette, taking the blood up immediately without drawing air in with it.

Hydrogen was obtained electrolytically, passed through alkaline pyrogallol, then through a solution containing  $\text{CO}_2$  in re-

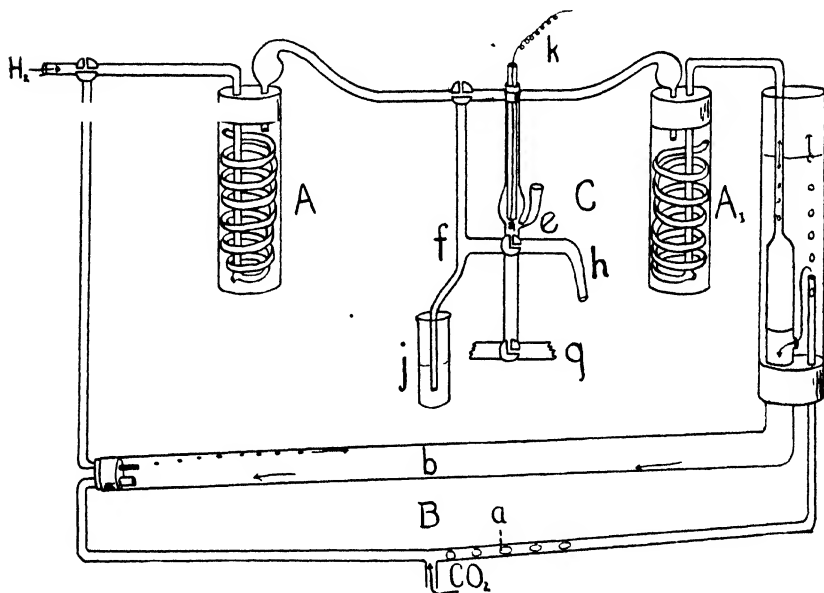


FIG. 2. Hydrogen electrode for small amounts of fluid, and apparatus for obtaining a given concentration of  $\text{CO}_2$  in hydrogen  $\frac{1}{4}$  size. For explanation, see text.

quired tensions, and finally through a bubbler containing sugar solution of the same osmotic pressure as the blood. The washer (Fig. 2, **A**) consists of a spiral coil made of 2 mm. tubing in a 20 cc. shell phial. The intake passes through a rubber cork to the bottom of the phial, at which point the wall of the tubing is blown and drawn out into a truncated cone shape. Solution entering the aperture here passes with the gases in alternate bubbles up through the spiral and out at the surface of the liquid,

and the gases are let off through a second tube with a catch bulb, inserted through the cork. These washers are very efficient, since each bubble of gas passes slowly through 12 inches of fine tubing, preceded and followed by a bubble of solution, which is renewed constantly and leaves a film even along the sides of the attenuated bubble of gas.

CO<sub>2</sub> was added to the H<sub>2</sub> as desired by passing streams of bubbles of each gas through different parts of the same solution of CaCl<sub>2</sub> (Fig. 2, *B*). CO<sub>2</sub> from a Kipp generator, passing up the narrow tube *a*, forces the salt solution around the system against the stream of H<sub>2</sub> bubbles passing up through the wide tube *b*, which is inclined just enough to make them move slowly, but the gases are not in contact except through the solution. The amount of CO<sub>2</sub> taken up by the H<sub>2</sub> depends chiefly on the concentration of CaCl<sub>2</sub> and slightly on the rate of flow of gases, *e.g.* saturated CaCl<sub>2</sub> gives about 1.5 per cent, half saturated 7.5 per cent, and the apparatus delivers constant proportions over long periods with constant rate of flow.

The hydrogen generator (Fig. 3) consists of a wide mouthed 500 cc. bottle *A* fitted with a wired-in rubber cork, through which passes a lead to the cathode *c*, a gas outlet to stop-cock *d*, a large bore stand-pipe containing the anode *a*, and a small glass tube *b*, leading from the bottom of the solution of 10 per cent NaOH and connecting by a rubber tube to a levelling bottle *B*. The latter is hung upon a coiled wire flexible spring, so adjusted that when H<sub>2</sub> accumulates in the generator and forces caustic solution over into the levelling bottle, the pressure does not increase appreciably, because the bottle lowers, due to added weight of caustic, enough to keep the difference in levels of the solution constant. As the bottle changes level, it runs a sliding contact *g* attached to it along a rheostat between 20 volts and zero, automatically regulating the rate of gas evolution. Iron electrodes are used, and the current is 110 volts d. c., only a part of which is led off by a potentiometer set up to the generator and regulator sliding contact.

A small electrode chamber was made from a four-way stop-cock whose arms had a bore of about 4 mm. (Fig. 2, *C*). A small enlargement was blown in one arm 1 cc. from the attachment to the cock, and a T-arm fused into the lower side of this bulb *e*.

The opposite arm *g* serves as a KCl bridge to the calomel electrode, one side arm *f* leading in  $H_2$ , the other *h* emptying the chamber without removal of the electrode. The latter, a short coil of platinum wire fused into a glass tube snugly fitting the chamber, is held in position by a split section of rubber tubing, fitting loosely enough into the flaring top of the chamber to

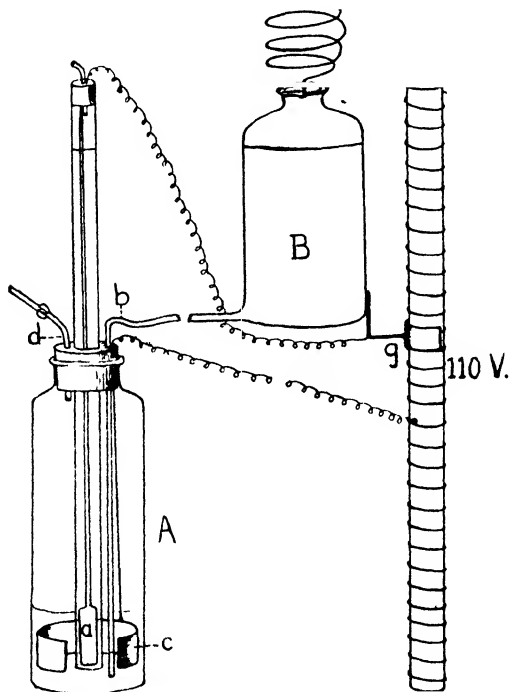


FIG. 3. Electrolytic hydrogen generator with regulating mechanism.  $\frac{1}{4}$  size. For explanation, see text.

allow escape of hydrogen (at *k*). *j* is an overflow and sampling tube.

In operation, the chamber is thoroughly gassed with the electrode in place, and solution added through the side tube which is sealed by oil following blood from the pipette. The electrode is then lowered into the solution until its glass shoulder drops below the edge of the bulb. Two or three bubbles of hydrogen<sup>4</sup>



with approximately the same tension of  $\text{CO}_2$  as the blood are then allowed to flow and lodge against this shoulder, in contact with the electrode, only the point of which touches the body of the solution. When more bubbles of gas are added for a second reading, the first are pushed past the electrode, and burst as they expand in the bulb. Using pure hydrogen on the one hand, and then hydrogen with considerably higher  $\text{CO}_2$  tension than the blood, on different samples of the same blood, it is found that the difference in pH readings at equilibrium is not more than 0.04 pH, indicating that the loss or gain of  $\text{CO}_2$  by the blood in contact with enough hydrogen to give a reading is very little, and is even less when the hydrogen used has approximately the tension of the blood to start with. Equilibrium once obtained is maintained for half an hour to within a millivolt (0.02 pH), indicating that the chamber, though not sealed at the top, protects the electrode and the solution in immediate contact with it effectively. Readings can be made on considerably less than 0.2 cc. of blood, though that was the amount usually used.

The rest of the apparatus is the conventional gas chain, Leeds and Northrup potentiometer, Weston standard cell, and high sensitivity galvanometer. Electrode were checked against a known buffer between readings, the buffer being checked with phenol red indicator by the drop ratio method, using Gillespie's tables for the pH intervals of the drop ratios, by which the buffer electrometric readings checked with the indicator readings as closely as the latter could be read. The normal calomel cell was checked against five other saturated calomel cells, which agreed with each other to within 0.2 millivolt, and the standard cell against a U. S. Government certified standard. Readings were made to the nearest millivolt (0.02 pH), which is reasonably within the accuracy of the other operations involved. The readings were made at room temperature, 24–25°; though the normal larval temperature is 33–34°, the data required in most of the experiments called only for comparative readings, and here the correction is not important. It has been figured approximately in a later section.

Potentiometer readings were converted into pH by the formula

$$\text{pH} = \frac{E \text{ observed} + E \text{ barometric correction} - E \text{ calomel cell (0.283 N)}}{0.00019837t}$$

Since in taking large samples of blood (1 cc.) for CO<sub>2</sub> determinations the latter part of the sample came from larvæ which had been exposed to the air outside the hive for some time and probably had lost some CO<sub>2</sub>, a number of small samples (0.2 to 0.3 cc.) were taken rapidly and the pH was measured directly. The average of these nine readings gave pH 6.83, ranging from 6.77 to 6.93. The higher values came from larvæ scattered in the comb, the lower from larvæ in combs where all the cells were filled, and the difference was apparently due to hive conditions and lower tension of CO<sub>2</sub> in the larvæ when adjacent cells of the comb were not occupied.

Several samples were equilibrated in the electrode chamber with pure H<sub>2</sub> for  $\frac{1}{2}$  to 1 hour, and the values of pH with practically all the CO<sub>2</sub> blown off rose to from 7.3 to 7.4. Further passage of H<sub>2</sub> through the chamber then resulted in no return of acidity after a maximum pH, indicating that during the time required for these and subsequent experiments no appreciable degree of autolysis or acid production took place in the blood. Other data from autolysis of blood *in vitro* (unpublished) also indicate that acid production in blood at neutrality is very slow. These data also give a value for the rising phase of CO<sub>2</sub> absorption curves from zero, as plotted on the blood chart (see below).

### *Oxygen Capacity.*

Oxygen determinations were made on samples for CO<sub>2</sub> content with the Van Slyke apparatus and technique, until it was demonstrated that the oxygen present was not appreciably more than would be physically dissolved. In blood from quiescent larvæ, before spinning of cocoons, the O<sub>2</sub> measured averaged 0.5 to 0.8 volume per cent. A sample equilibrated with air and CO<sub>2</sub> at blood tension was analyzed for O<sub>2</sub> by Dr. Doisy and found to contain 0.7 volume per cent, which is probably within the experimental error of the amount physically dissolved. Larvæ that were spinning ran consistently lower than this, often having so little oxygen that its detection was uncertain; certainly as low as 0.2 volume per cent. The oxygen tension in the hive air is never normally below 15 per cent, but the spinning larvæ are sealed over with a wax cover, which, though porous, must slow diffusion, and the marked increase of CO<sub>2</sub> is evidence that con-

siderable work is done in spinning, and data (unpublished) from respiratory calorimeter experiments indicate that at this time the oxygen consumption increases. The increased consumption and decreased diffusion rate should explain the low values for spinning larvæ.

Muttkowski (1921) finds in a number of aquatic insects copper in amounts proportional to that in the blood of crustaceans, which are known to contain hemocyanin. He also mentions finding copper in bees and other insects, and in plants, but does not state the amounts. Certain chironomid larvæ are known to contain hemoglobin. The oenocyte cells of insects, which develop numerously at or before pupation and disappear as pupation proceeds, have been connected with the oxygen metabolism, but the data are not conclusive. If copper in bee larvæ enters into a respiratory compound, its amount would seem to be very slight.

### *CO<sub>2</sub> Capacity and Exchange.*

*Method and Calculation of Experimental Error*—CO<sub>2</sub> content of the blood was determined both on samples as taken and on samples equilibrated with different tensions of CO<sub>2</sub>. Since no oxygen carrier was present the effect of oxygen unsaturation need not be considered, but as a precaution two samples were equilibrated with air and CO<sub>2</sub>, and agreed both in pH and in carbonate content with samples equilibrated with H<sub>2</sub> and CO<sub>2</sub> within the limits of accuracy of the experiments. This gives a second line of evidence that no O<sub>2</sub> carrier is present.

The method employed was as follows: About 1 cc. of blood was drawn in a pipette under oil, and on 0.2 cc. the pH was determined electrometrically. Half of the remainder was equilibrated with a known tension of CO<sub>2</sub> by bubbling H<sub>2</sub> (or air) plus CO<sub>2</sub> through it in a narrow test-tube for 20 to 30 minutes at room temperature, 25°. Results showed later that this did not bring about equilibrium with tensions that were considerably lower than the initial blood tension. The rest of the sample was used for a CO<sub>2</sub> determination in a Van Slyke apparatus by the usual technique, with the corrections of Van Slyke and Stadie (1921). The equilibrated sample was again divided, a minimal amount being used for a final pH determination, and the rest

for  $\text{CO}_2$ . Samples were done in pairs; taken as nearly as possible under the same conditions, and equilibrated, one to a high tension, and one to a lower tension than that of the blood as drawn. One complete set of determinations gave two points on a curve for which  $\text{CO}_2$  tension,  $\text{CO}_2$  content, and pH were measured, and two points for which pH and content alone were measured. The determination of  $\text{CO}_2$  as drawn on the second sample was omitted in some cases, giving only three points, but a larger sample of blood to equilibrate. The points plotted were: pH as measured and total volume per cent  $\text{CO}_2$  on a chart, the pH vectors of which were calculated for  $\text{pK}_1 = 6.1$ , the combined  $\text{CO}_2$  ordinates for the pH points being increased by the amount of dissolved  $\text{H}_2\text{CO}_3$  corresponding.

From twelve determinations of total  $\text{CO}_2$ , pH, and  $\text{CO}_2$  tension obtained, an attempt was made to calculate Hasselbalch's constant  $\text{pK}_1$  as a check on the determinations in general, and for the drawing of the pH vectors of the chart. Warburg (1922) has found that this factor varies with temperature, salt concentration, and possibly with sugar concentration, and the high osmotic pressure of this blood renders its value problematical. Since in these computations the tension as measured on the low tension experiments was undoubtedly too low, this would make the constant as calculated from these experiments too low. The data were, therefore, divided into two groups, the six with the lowest tension in one group, and the six with highest in the other. From the formula,

$$\text{pH} = \text{pK}_1 + \log \frac{\text{NaHCO}_3}{\text{H}_2\text{CO}_3}$$

$$\text{pK}_1 = \text{pH} - \log \text{NaHCO}_3 + \log \text{H}_2\text{CO}_3$$

but

$$\text{Volume per cent H}_2\text{CO}_3 = \frac{P}{760} 100\alpha$$

and

$$\text{NaHCO}_3 = \text{total CO}_2 - \frac{P}{760} 100\alpha$$

where  $\alpha$  is the absorption coefficient of the larval blood at  $25^\circ$  and is unknown. It can be approximated as follows:

Relative absorption coefficient of human blood serum, $\alpha_s$	= 0.975 (Bohr's value)
Solubility coefficient of $\text{CO}_2$ in water at $25^\circ$ , $\alpha_{\text{H}_2\text{O}}$	= 0.76
Solubility coefficient (approximate) of bee blood at $25^\circ$	= $0.975 \times 0.76 = 0.74 = \alpha_x$

The combined  $\text{CO}_2$  calculated with this value gives very little error in pH or  $\text{pK}_1$ , but the same error in the dissolved  $\text{CO}_2$  in the denominator of  $\frac{\text{NaHCO}_3}{\text{H}_2\text{CO}_3}$  would have a greater effect, since  $\log \text{H}_2\text{CO}_3$  changes more rapidly than  $\log \text{NaHCO}_3$  with change of  $\alpha$ . This factor  $\alpha$  can then be put into a new constant  $\text{pK}_x$ , where

$$\text{pH} = \text{pK}_1 + \log \text{NaHCO}_3 + \log 760 - \log p - \log 100\alpha_x$$

whence,

$$\begin{aligned}\text{pK}_x &= \text{pK}_1 + \log 760 - \log 100\alpha_x \\ &= \text{pH} - \log \text{vol per cent NaHCO}_3 + \log p \text{ mm Hg}\end{aligned}$$

and the value  $\text{pK}_x$  should be more constant than  $\text{pK}_1$  if there is an error in  $\alpha_x$ .

The first group of data (low  $\text{CO}_2$  tension) gives a value of  $\text{pK}_x$  of 6.99 with a mean variation of 0.10. The second group gives a value of 7.08, with a mean variation of 0.04. Assuming the value of 0.74 again for  $\alpha_x$ ,  $\text{pK}_1 = \text{pK}_x - 1.01$ , giving values of 5.98 and 6.07, approximately. The last figure is certainly the more nearly correct, and in drawing the charts the value 6.1 was made use of. The curve near 0  $\text{CO}_2$  tension was made asymptotic to pH 7.4, in accordance with pH measurements on blood equilibrated with pure hydrogen, as noted in a previous section.

Points connected by straight dotted lines represent one set of data. Circles represent the locus or range of values of unsealed larvæ, spinning larvæ, and pupæ, respectively.

Finally in interpreting these values, account should be taken of the fact that the blood was equilibrated and the pH measured at  $25^\circ$  instead of at  $34^\circ$ , the larval temperature. This affects the value of  $\alpha$  and of the dissociation factor in  $\text{pK}_1$ , and hence the combined and dissolved  $\text{CO}_2$  and  $p$ , and thence pH. The significant differences, however, are small, while the relative values

for larvæ at different stages and conditions are not altered. An approximate correction may be arrived at by a consideration of the Hasselbalch equation.

Assuming a certain value of the Na ion and CO<sub>2</sub> tension, equilibrium between the HCO<sub>3</sub> ion and other ions competing for sodium should shift but little with change in temperature, and the combined NaHCO<sub>3</sub> should be practically constant at constant CO<sub>2</sub> tension. However, the three states, combined CO<sub>2</sub>, dissolved CO<sub>2</sub>, and tension CO<sub>2</sub>, remain in equilibrium, and a rise in *t* will reduce the quantity of dissolved CO<sub>2</sub>, increasing the combined CO<sub>2</sub> by the amount of the reduction, and the tension proportionally. Assuming the solution to contain the same quantity of total CO<sub>2</sub> at two temperatures, there will be little error in assuming that the ratio  $\frac{\text{combined CO}_2}{p}$  is constant. However,

$\frac{100p}{760}\alpha$ , i.e. the dissolved CO<sub>2</sub>, will change with change in  $\alpha$ , and the ratio  $\frac{\text{combined CO}_2}{\text{dissolved CO}_2}$  will increase with increase in *t* by a factor  $\frac{\alpha_{t_0}}{\alpha_t}$ . Warburg (1922), working with bicarbonate solutions at 18

and 38°, found that pK<sub>1</sub> varied with the temperature by a value of  $-0.007t$ , a decrease of about 0.15 for a 20° rise, due to change in the dissociation constant of H<sub>2</sub>CO<sub>3</sub>. For the conditions of the experiments considered in this paper, pK<sub>1<sub>34°</sub></sub> = pK<sub>1<sub>25°</sub></sub> - 0.065, and

$$\alpha_{25^\circ} = \alpha_{34^\circ} \times -1.26 \text{ or } \log \frac{\alpha_{25^\circ}}{\alpha_{34^\circ}} = 0.10.$$

$$\text{pH}_{25^\circ} = \text{pK}_1 + \log \frac{(\text{NaHCO}_3)_{25^\circ}}{(\text{H}_2\text{CO}_3)_{25^\circ}}$$

$$\text{pH}_{34^\circ} = \text{pK}_1 - 0.065 + \log \frac{(\text{NaHCO}_3)_{25^\circ}}{(\text{H}_2\text{CO}_3)_{25^\circ}} + 0.10$$

whence,

$$\text{pH}_{34^\circ} = \text{pH}_{25^\circ} + 0.035, \text{ or } \text{pH}_t = \text{pH}_{t_0} + \log \frac{\alpha_{t_0}}{\alpha_t} - 0.007t$$

This value is probably within the experimental error of these experiments, but is a systematic one, and should, therefore, be

added to all the pH values.<sup>1</sup> The total CO<sub>2</sub> values have no correction, and the tension should be higher than the values on the chart by a value proportional to the increase in combined CO<sub>2</sub> with *t*; *i.e.*,

$$\frac{p_{25^\circ}}{p_{34^\circ}} = \frac{\text{combined CO}_2_{25^\circ}}{\text{combined CO}_2_{34^\circ}} = \frac{\text{total CO}_2 - \text{dissolved CO}_2_{25^\circ}}{\text{total CO}_2 - \text{dissolved CO}_2_{34^\circ}}$$

The ratio of the combined CO<sub>2</sub> to the dissolved for this blood at 25° is about 5 to 1, so that the ratio

$$\frac{\text{Combined CO}_2}{\text{Dissolved CO}_2} \text{ at } 34^\circ = \frac{6 - 0.79}{0.79} = \frac{5.21}{0.79} = \frac{6.6}{1}$$

and

$$\frac{p_{25^\circ}}{p_{34^\circ}} = \frac{5}{5.21}, \quad p_{34^\circ} = p_{25^\circ} \times 1.04$$

The correction for CO<sub>2</sub> tension would, therefore, appear to be insignificant compared with the variations in tension that occur under natural conditions.

<sup>1</sup> Hasselbalch (1917) calculated in bicarbonate solutions at 18 and 38° that the pH increased 0.12 with 20° increase of temperature, if the CO<sub>2</sub> tension was constant. He used -0.10 as the correction for 20° for pK<sub>1</sub>, or -0.005 per degree. In blood serum as reckoned from his curves, there was an increase of 0.10 pH for 20° rise, but no appreciable change in the pH of whole blood, the hemoglobin presumably compensating for the change expected by a change in the dissociation constant of oxyhemoglobin. Taking Warburg's correction of -0.15 pK<sub>1</sub> instead of -0.10, the change in serum would be about -0.07 pH for 20°, or -0.0035 per degree, in this temperature range. This would give a value of about -0.0315 for 9° for bicarbonate or serum from 25-34°, which is about the middle of the range. Since  $\alpha$  does not vary constantly with the temperature, the correction for  $\frac{\text{NaHCO}_3}{\text{H}_2\text{CO}_3}$  at higher temperatures would be less than at lower. Taking into account that *p* would rise at higher temperature with rise in combined CO<sub>2</sub> if the total CO<sub>2</sub> were constant, a slightly larger value than this for increase of pH in blood without cells would be obtained, computed on the basis of total CO<sub>2</sub> at the higher temperature. Barach, Means, and Woodwell (1922), in making corrections for pH with change of temperature in whole blood, not only took too large values for change in  $\alpha$  (assuming  $\alpha$  varied constantly with change in temperature, and that its curve was a straight line), but also ignored the correction necessary for pK<sub>1</sub>, which almost completely compensates for this change in  $\alpha$  at higher temperatures in serum, and they did not consider at all the change in the dissociation of oxyhemoglobin that apparently compensates for the rest of the change in  $\alpha$ .

*Absorption Curves (Fig. 4).*—Curve I is drawn from two sets of data; it consists of four readings from two samples of blood, one of each as drawn, and one of each equilibrated, and of three readings from another pair of samples, one as drawn and two equilibrated. The middle points represent the blood as drawn.

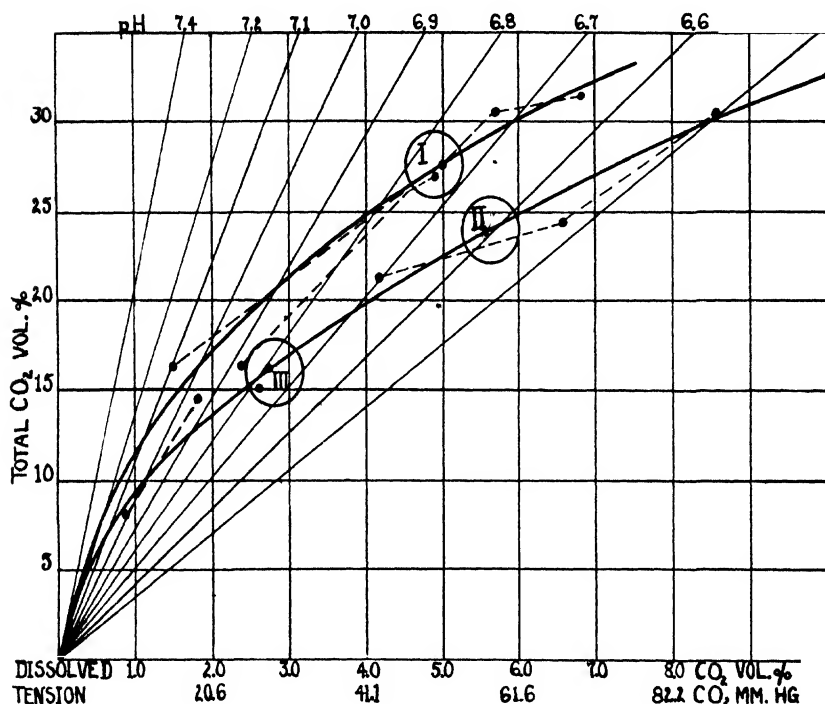


FIG 4 CO<sub>2</sub> absorption curves for blood of larvæ and pupæ of the honey bee. Normal range for pH and total CO<sub>2</sub> indicated by large circles. Further explanation in text.

Measured pH and total CO<sub>2</sub> were plotted as described heretofore. It represents average normal values.

Curve II consists of three readings from two samples from larvæ that were sealed over and spinning cocoons, but otherwise under the same conditions as Curve I. The middle value is of blood as drawn, the other two equilibrated. Two other points are plotted for low tensions from another set of samples.



The change from a condition as in Curve I to that of Curve II is a continuous one, though rapid, and these curves represent the extremes.

At Curve III have been plotted values from pupæ which were quiescent, after spinning. At this stage equilibration with  $\text{CO}_2$  cannot be brought about satisfactorily, because the blood contains large amounts of autolyzing cell debris, which rapidly produce acid upon removal. The values represent determinations on blood as drawn.

*CO<sub>2</sub> Content of Blood as Drawn.*—Taking all values of blood as drawn from full grown bee larvæ, the range of ten samples is from 26.1 to 34.6 volumes per cent, with two other samples, 22.4 and 24.7 from larvæ in an outside comb of the hive, with relatively few larvæ scattered in the cells. The average is 29.2 volumes per cent. Taking all values for larvæ sealed in the cells during the spinning of the cocoons, five samples, the average is 23.3, ranging from 21.6 to 24.9 volumes per cent, with one other sample which was from larvæ that had just been sealed in, and which had a value of 26.2 volumes per cent. The values for pupæ lie around 15 volumes per cent of  $\text{CO}_2$ , but at this stage constituents have been added to the blood from histolyzing tissues. The pH as measured for this stage indicates, however, that the combining power of this mixture of blood and tissue debris is not very different from that of the larvæ that are spinning.

With three exceptions, all the larvæ were drones or male larvæ, which are larger than the worker or female, and yield more blood per larva. The three determinations on worker larvæ showed no significant differences from those on drones. About thirty larvæ were required for 1 cc. of blood, which took about half an hour to obtain, and in all about 35 cc. of blood were worked up in these experiments. Variations in the values obtained for measured  $\text{CO}_2$  are only partly to be assigned to experimental error of the apparatus, the use of small samples, and loss of  $\text{CO}_2$  in drawing the blood (though the second sample, drawn an hour or two after the first from the same material, generally read from 1 to 3 volumes per cent lower); a real difference in values may be due to the fact that the distribution of larvæ in the comb allowed different diffusion rates between the blood and hive air (see below).

*CO<sub>2</sub> Tension in the Hive Air.*—The CO<sub>2</sub> tension in the bee hive is low, and is maintained remarkably constant. Data from April and May, 1922 and 1923, show variations of from 0.41 to 0.56 per cent (3.1 to 4.2 mm. Hg). If the entrance to a hive is closed, distress is soon evidenced by loud humming, and upon opening again, the bees rush out and cling to the side of the hive, fanning their wings violently. If a small colony, *i.e.* one with few bees, is closed in this way, the disturbance follows, if at all, only after considerable time. If the entrance is kept closed in hot weather, the bees move about violently inside the hive, raising the temperature by their activity until the death of the colony may result. Bees also become excited due to confinement with wire screen over the entrance, but the symptoms of distress are not nearly so pronounced, nor is the behavior so characteristic on their release. After partial smothering they are little inclined to sting, while after simple confinement their annoyance is evidenced by a more vindictive temper. The ventilation after confinement is perhaps partly for the purpose of cooling the cluster, but seems to be also for the purpose of lowering the CO<sub>2</sub> tension.

Readings of CO<sub>2</sub> tension were taken on a hive of bees on a bright day, May 4, with many bees flying, temperature 20°C. outside. The hive was then opened, three frames, adjacent to the tube from which the air was drawn, were removed, then the frames were replaced and the hive was closed, and a further series of readings taken. The tube through which samples were withdrawn penetrated to the middle of the cluster, between two frames of unsealed larvæ. Fig. 5 gives the values of CO<sub>2</sub> tension in the hive for the next hour and a quarter.

The rise in CO<sub>2</sub> in evidence, as soon as the first reading could be made, was obviously due to increased activity due to the disturbance. The increased ventilation, which in this case may have been for the purpose of warming the hive, or for the removal of CO<sub>2</sub>, reduced the CO<sub>2</sub> after nearly an hour to slightly below the initial value, after which it rose to the customary level.

This experiment indicates that the changes in the hive tension due to removal of larvæ for blood were not great enough to have any appreciable effect on the blood tension of the larvæ after they were returned to the hive, though the increased ventilation inside the hive itself may have resulted in a more efficient

removal of gases from their immediate vicinity. This might account for the lower tension in the second samples of blood, since this increased ventilation appears to last for an hour or so. The larvæ also presumably lose  $\text{CO}_2$  while out of the hive, and do not reach their former level for some time after returning. Lowering of metabolism due to lowering of temperature may also affect the  $\text{CO}_2$  content, since the room temperature in which the larvæ were worked on was below the hive temperature.

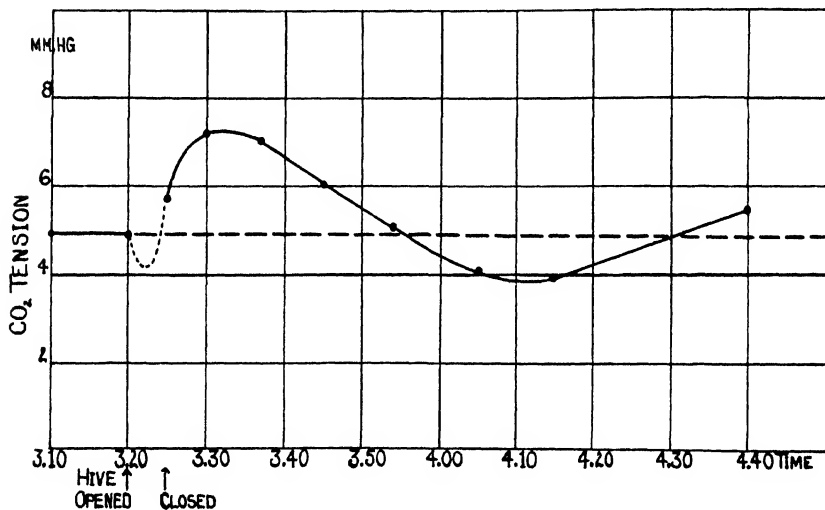


FIG. 5. Fluctuation in  $\text{CO}_2$  tension in bee hive, due to disturbance. Explanation in text.

A more significant factor, however, in determining the  $\text{CO}_2$  in a given sample is the distribution of the larvæ in the combs. When every cell of a given area has a larva in it, both sides of the frame, the respiratory gases can escape only from the outer surface, since all other surfaces are adjacent to other larvæ. When, however, only scattering cells have larvæ in them,  $\text{CO}_2$  may diffuse through the wax partitions and escape from the empty cells. This hypothesis checks with the findings on  $\text{CO}_2$  content.

*CO<sub>2</sub> Production per Gm. of Weight.*—Calorimetry experiments on larvæ and pupæ of various stages (unpublished data) indicate

a marked increase of  $\text{CO}_2$  given off and also of  $\text{O}_2$  utilized during the spinning of the cocoon, followed during metamorphosis by a decrease to below the larval level, until, when the insect quickens before emerging, the  $\text{CO}_2$  increases again. Respiratory quotients for the pupal stage (also determined by Krogh and co-workers) fall remarkably low (0.65), implying a consumption of oxygen greater than sufficient to burn fat during pupation, or a lower  $\text{CO}_2$  production than would be expected from the basal metabolism. The  $\text{CO}_2$  production curve must be corrected to represent the true basal metabolism, though it gives a rough measure of it. For instance, during spinning the  $\text{CO}_2$  tension of the blood increases, but its content decreases, giving an abnormally high  $\text{CO}_2$  output. Acid produced by muscular activity apparently appropriates some of the alkali reserve of the blood. During spinning the larva ceases to receive food (largely carbohydrate) from the intestine, and commences to utilize its stored food reserves, in which fat is a major element, changing the  $\text{CO}_2$  output for the same activity. The magnitude of these corrections cannot be determined without more work on the fat-carbohydrate metabolism, which is now in progress. The increase in  $\text{CO}_2$ , however, still seems to be a real one, due to muscular activity added to the basal activity of the quiescent larva or pupa, and the result is to accentuate the increase of blood  $\text{CO}_2$  tension which would be brought about by sealing the larva over in the cell.

Three factors may thus be inferred to increase the blood  $\text{CO}_2$  tension; decreased capacity due to products of muscular activity, decreased diffusion rate due to the wax cover, and increased  $\text{CO}_2$  production. The combined result is an increased acidity at a time when the change from a carbohydrate to a fat diet might be inferred to predispose the larva to a further acidosis, especially under the reduced oxygen tension which the analyses show. Acidosis from a high fat diet, from muscular activity, and from high  $\text{CO}_2$  tension would thus tend to reinforce each other and furnish in the blood at the beginning of metamorphosis the condition most suitable for the autolysis of larval tissues. As has been shown elsewhere (Bishop, 1922), this autolysis, common to all insects, is in the bee larva an extremely rapid one, and sets in very abruptly after or even during spinning.

*Changes in CO<sub>2</sub> and pH in Metamorphosis.*

From the smoothed curves of equilibrated blood, and from the data on CO<sub>2</sub> contained in larval blood as drawn, and the pH as measured, the normal values of pH, total carbonate, and CO<sub>2</sub> tension may be assigned to larval blood as follows:

For mature larvæ before metamorphosis, total CO<sub>2</sub>, 25 to 30 volumes per cent; tension CO<sub>2</sub> in blood, 40 to 55 mm. of Hg; and pH 6.75 to 6.85. When but little brood is present in the combs, the CO<sub>2</sub> may be reduced to the lower values, but the normal for vigorous condition of the colony is around 28 mm., pH 6.8.

For larvæ that have been sealed in their cells and are spinning cocoons, the total CO<sub>2</sub> is lower, 22 to 25 volumes per cent, the tension in the blood 50 to 60 mm. of Hg, and the pH 6.60 to 6.65. Since the CO<sub>2</sub> production of this stage is increased, as evidenced by high tension and increased output, the difference in combining power must result from the increase in the products of activity; *i.e.*, muscular work in spinning.

For pupæ, while no absorption curves have been plotted, for the reason given above, the values of total CO<sub>2</sub> and pH indicate that such a curve would lie as low as that for the spinning larvæ. Since here CO<sub>2</sub> production has decreased again, the low combining power must result from further products of metabolism other than muscular activity, possibly autolysis of larval tissues. In the mean time, the total CO<sub>2</sub> contained has decreased from the amount in the blood of the larvæ that are spinning, due to decreased CO<sub>2</sub> production with quiescence of the pupa. This, with decreased combining power, indicates decreased tension of CO<sub>2</sub>. The result is that the pH is allowed to rise slightly in spite of the acid production inferred from low combining power, almost to the initial larval figure. From here on the reaction of the body fluid must be a balance determined by the rate of autolysis of larval tissues and the rate of synthesis of the pupal tissues, which are disposing by their metabolism of the products of larval tissue autolysis; the whole tending to reach and maintain an equilibrium depending on the buffer action of the blood.

The question arises whether this cycle throws any light on the causative mechanism of metamorphosis, or in general, of histogenesis and development. The imaginal insect tissues develop

only after the larva has grown, but from elements present throughout larval life. Could a slight change in pH, due to a change in the carbohydrate-fat metabolism and accentuated by increased CO<sub>2</sub> tension, be one of the direct causative factors in stimulating development of these dormant tissue elements? Or does it only operate, through encouraging autolysis, to furnish nutriment for these growing tissues? The objection must be met that one set of tissues does develop to larval maturity under one set of circumstances, while the other does not. The cytology of the autolyzing cells indicates an elaborate internal mechanism which, however activated at the beginning of metamorphosis, proceeds further in a more or less predetermined manner. Also some insects, *i.e.* wax worms and blow fly larvæ, live, as larvæ, on a high fat diet, though the possible carbohydrate and protein content of such a diet has not received proper attention. At present the data are far from conclusive either way.

It may be reasoned that if in a body fluid of a given reaction, one set of tissues histolyzes while another set develops, either the tissues must differ fundamentally in their demands, *i.e.* in their metabolism, and that "protoplasm" is itself capable of metabolizing at different reactions, or else that cells have the capacity of maintaining their own "protoplasmic" pH against that of the environment, *i.e.* the body fluid, and are more or less independent of slight changes in reaction. The reaction of the medium then might be a determining factor in the exchange of materials between cell and medium; that is, might affect the direction or end-point or rate of a system tending toward equilibrium, without affecting markedly the reaction of the cell itself. Further, embryonic tissue may differ in this respect from tissues, even growing tissues, that are fully differentiated. Certainly the early embryo in an egg before complete differentiation lacks the excretory mechanisms for disposing of waste that a more developed organism has, and would appear to react differently to the accumulated products of breakdown of the material it feeds upon, which generally contains considerable lipoid and fat.

Metamorphosis and concomitant larval histolysis or autolysis take place prematurely in many insects, including the bee larva, if the food supply is cut off, a smaller but perfect insect being developed. The cessation of nutriment would, therefore,

seem to be one of the causative factors of the insect's reverting to what is essentially a second embryonic stage. In the bee larva metamorphosis comes on more abruptly and proceeds more rapidly than in most insects. The factors in the metabolism of the bee larva that may be correlated with this abruptness of transformation are those which would increase acidosis and autolysis; relatively high temperature, high  $\text{CO}_2$  tension, low  $\text{CO}_2$  capacity, reduced oxygen tension, change from a diet rich in carbohydrate to one rich in fat, confinement in a wax cell which hinders respiratory exchange, and muscular activity in spinning that is relatively violent for an insect with this larva's muscular development. It is suggested that change in the metabolic equilibrium, whatever the ultimate cause, is the immediate cause of tissue autolysis in this case, and the secondary factors tending to bring about a more acid pH are contributing causes that accelerate the process in those insects where and to the extent that they obtain. The relation of these events to the initiation of development of the pupal tissues cannot be inferred from the data at present available.

#### SUMMARY.

1. Specific gravity of mature larvæ of worker honey bees is 1.045.
2. The osmotic pressure of worker larval blood in terms of freezing point lowering gives a value of  $-0.86^\circ\text{C}.$ , and the osmotic pressure decreases during pupation.
3. The pH of larval blood (measured at  $25^\circ$ ) is near 6.8, varying with conditions in the hive.
4. The oxygen capacity of larval blood is within a reasonable error the amount that could be physically dissolved, and no evidence of a chemical carrier is found. The content decreases during spinning, when the larva is enclosed in a cocoon, because diffusion is cut down while consumption increases.
5.  $\text{CO}_2$  absorption curves indicate that the blood (of drone larvæ) decreases in  $\text{CO}_2$  capacity and content during spinning of the cocoon, but the tension of  $\text{CO}_2$  increases, and the H ion concentration increases, giving evidence of loss of alkali reserve through the production of acid other than  $\text{CO}_2$ . After spinning, pupal blood shows a decrease of  $\text{CO}_2$  tension and content, with

little change in capacity, allowing the pH to return to approximately the initial level.

6. Variation in CO<sub>2</sub> tension and, therefore, in CO<sub>2</sub> content and pH of blood from larvæ in the same stage may be assigned to variable distribution of larvæ in the cells of the comb, affecting diffusion of respiratory gases; but not appreciably to variation in the tension of CO<sub>2</sub> in the air of the hive, which is maintained at a low and constant level.

7. The acidosis that occurs during spinning appears to be one of several factors present that should encourage autolytic changes in a tissue. Such changes do, in fact, take place in the bee larva. High concentration of the H ion may, therefore, be assigned as a contributing cause to the rapid metamorphosis of this insect.

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changes in the pH and total acid content at the beginning of pupation in the bee larva, producing a condition favorable to autolysis. The CO<sub>2</sub> tension rises and the buffer value and pH fall as the larva is enclosed in its cocoon, due to muscular activity of spinning and to decrease in diffusion rate of respiratory gases. In the present paper is undertaken an investigation of autolysis *in vitro* of the larval and pupal tissues of the honey bee, in an attempt to determine whether autolysis *in vivo* may be assigned to changes in acidity, to changes in enzyme content, or to cessation of food supply and demands for nutriment on the part of the pupal tissues, or whether the cytological development of the fat body cells predetermines some such change more or less independently of the above conditions, as the elaborate and complicated cell phenomena might imply.

#### *Technical Procedure.*

Both fat and protein autolyses were attempted. The material was prepared as follows:

To obtain the fat body tissue free from other constituents, the larvæ were placed under water on filter paper, and the heads were cut off and the blood was allowed to escape. Into the cut end of the larva the point of the scissors was inserted, and the larva pulled onto it with forceps, and slit along the mid-dorsal line, the scissor point following the hemal space between the intestine and the heart. The contraction of the muscles of the body wall drew the larva open. The cut anterior end of the intestine was then seized with fine forceps, and torn carefully from the underlying fat body back as far as the hind intestine. This was seized, and cut at its posterior end; by carefully lifting, the malpighian tubules could then be drawn from among the fat cells. On either side of the body, embedded in the fat tissue, but not completely covered by it, the convoluted spinning glands extend from the head nearly to the posterior end of the larva. By careful manipulation these could be lifted out intact by seizing them near the posterior end. The laminated lobes of fat tissue then projected from the body wall up into the water, and could be washed reasonably clear of blood by a gentle stream of water from a pipette. The remaining mass was laid open side downward on the filter paper in the bottom of the pan.

When a sufficient number had been thus prepared (10 to 50), the filter paper was gently lifted out and drained by placing on a pile of filter paper under a watch crystal. The drained tissue was scraped into a watch crystal of known weight, and the weight of the tissue determined. It was then well shaken with water in a test-tube, strained through two thicknesses of fine cheese-cloth, into a graduate, the residual body tissues were shaken again with more water and strained as before into the first lot. The tissue which did not pass the cheese-cloth was again dried loosely on a filter paper, and weighed, and the weight of the tissue carried by the water determined. The graduate was filled to a quantity which was a known multiple of the weight of the tissue, giving a solution of the desired concentration; and an excess of toluene was added, the whole poured into a narrow mouthed flask and autolyzed at 40°C. From the bottle samples were drawn as desired, diluted, neutralized to phenolphthalein, treated with freshly neutralized formaldehyde, and titrated for amino-acids with 0.02 N alkali from a burette. Burettes were selected whose tips produced even sized drops, regularly timed, and the number of drops required to neutralize the material of the test was taken as a measure of acidity. Knowing the drops per cc. at a given rate of flow—the burette was left running during the titrations, at the rate of about 2 drops per second—the acidity may be computed from this record.

Tissues from larvæ and pupæ of different ages were tested, but chief reliance is put upon those experiments involving larvæ which had attained nearly full size but were still eating and were about to be sealed over, and pupæ which had straightened out in the cells and whose heads were just forming. In the former the fat body was large, and easily separated from the other tissues, but the nuclei of the cells were still intact. In the latter, the cells contained the later stages in the formation of albuminoid globules, and were about ready to break loose into the blood stream. From still later stages of pupæ it was difficult to free the tissues from blood and other cellular elements of the developing pupa, and the yield of tissue was less, and the exact amount of fat body tissue uncertain. In early larval stages it was difficult to separate the fat body tissue from the other tissues.

*Protein Autolysis.*

To determine roughly what tissues would autolyze most readily, and to what relative extent, preparations were made of the fat body tissue alone, of blood alone, of fat body and its accompanying blood, of the residual body tissues left after separating out the fat body material, and of testis. It is impossible, without elaborate analyses and dry weight determinations of these tissues, to reduce all the solutions to a common dilution; the residual tissues, for instance, certainly contain considerable chitin which is inert, and the water content of blood and fat body is different. These relative determinations are, therefore, approximate.

To reduce the autolysis curves to comparable terms, they were plotted with the time in days as ordinates, and as abscissæ, the ratio of the volume of alkali neutralized to the dry weight of the sample, *i.e.*, the amino-acid per gm. of dry tissue. That is, they were plotted as if all runs were of the same dilution. For comparison, certain curves were also plotted with abscissæ figured as ratios of amino-acid to cubic centimeters of autolysis solution.

*Data.*—Fig. 1 shows certain of these curves, those numbered 1' to 6', and 7 and 8 plotted in terms of autolysis per cc. of solution, those numbered 1 to 6, in terms of autolysis per gm. of dry weight in these solutions. Curves 1 and 2, 3 and 4, and 5 and 6 show autolyses, the first and second members of each pair of which contained tissues from pupæ and from larvæ, respectively, taken just before spinning and just after metamorphosis to pupæ. Each of these pairs of solutions was treated alike and tested at the same time. Of these, Nos. 1 to 4 were of worker pupæ and larvæ, 5 and 6 of drone. Curves 3 and 4 show corrections for marked differences in dilution, indicating that variations in water content did not affect the results materially. Curve 7 shows autolysis of the residual body tissues strained out of that used for Nos. 5 and 6, and Curve 8 is of testis material from drone pupæ with testes in almost fully developed condition. For their interpretation the data in Table I are pertinent.

*Discussion.*—It may be stated in the first place that the fat body tissues autolyze readily; that the residual body tissues

autolyze but little, and the testes practically none. Tissues autolyzed with and without the accompanying blood, and diluted by the same factor of weight, autolyzed at approximately the same rate and to the same relative extent. Blood alone autolyzed but slightly. As for the residual body tissues, what little

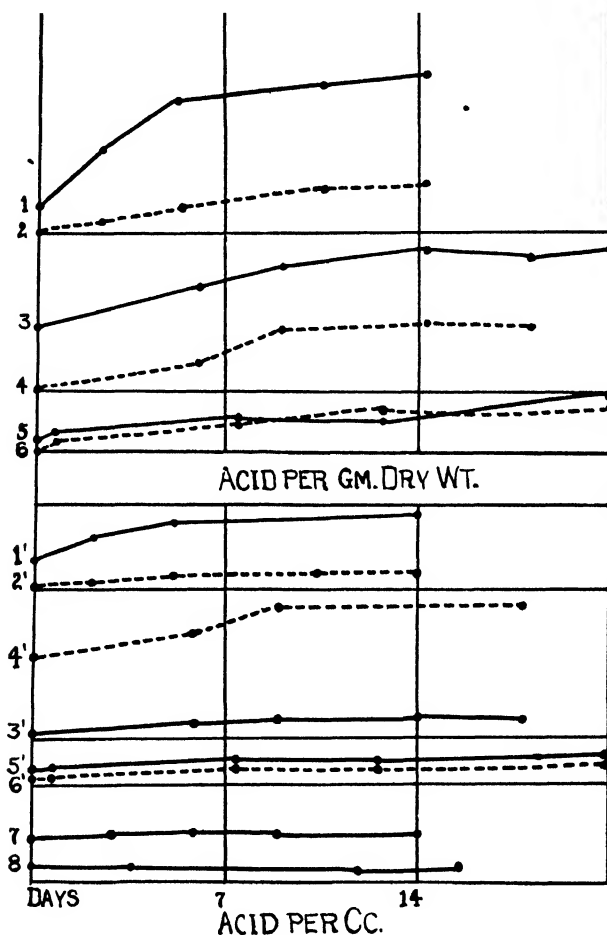


FIG 1. Unbuffered autolyses of bee larval and pupal tissues. Abscissæ represent protein autolyses, ordinates, time in days. Curves 1' to 6', and 7 and 8, autolysis per cc. of solution; Nos. 1 to 6, autolysis per gm. of dry weight. Further explanation in text.

autolysis occurred may probably be assigned predominantly to the fat tissue which could not be perfectly separated out from them, for considerable of their bulk was chitin which must be considered inert; and the other body tissues must, therefore, autolyze but slightly. It is safe to conclude that for the body tissues the curve is exaggerated. In the testis the amount of autolysis was within the limits of experimental error; the changes in level recorded representing in this case a variation between 6 and 4 drops of 0.02 N alkali (or 0.044 cc.) in 1 cc. of solution.

TABLE I.

*Data on Fat Body Tissue Employed in Autolysis.*

	Pupæ	Larvæ.
Weights, each average* (worker), gm	0 135-0 140	0 140-0 150
Average fat body tissue	0 08	0 07
" blood		0 04

\* The fact that more tissue can be obtained from pupæ than from the larvæ of greater weight is not an indication of a greater amount of tissue in the pupæ. Not all the cells can be shaken free from the tissues of either stage, but the larval tissues adhere to the other structures more firmly. The fat body probably comprises 60 per cent by wet weight of the whole larva, the blood 25 to 30 per cent. The amount of blood in the pupæ is more difficult to determine when the tissues tend to disintegrate, as blood is drawn, but it seems to become considerably less in amount as pupation goes on, the emerging insect having relatively little. The large amount of blood in the larvæ comprises a water reserve for pupation.

Since each curve represents the autolysis of tissues taken from a number of different larvæ or pupæ (10 to 50), the tissue may be considered to average fairly uniformly for the different autolyses, and the individual differences usual in autolyses of the same organs from different animals may be ignored. In each of the pairs of larval and pupal tissues figured, and in others not shown, the difference in extent of autolysis is in the same direction; the tissues from the pupæ autolyze more rapidly and more completely than those from the larvæ; and the difference in each case is more pronounced than the irregularity of the curves or the probable error of the experiments.

*Protein Autolysis at Controlled pH.*

The previous series of experiments gives little evidence as to the cause of the difference in autolyses of larval and pupal tissues. The acidity of the mixtures was not determined quantitatively, though it was observed that they became increasingly acid in rough proportion to the degree of protein autolysis. To determine the effect of the reaction on the autolytic process the following procedure was adopted.

Tissues were dissected out as above, from worker larvæ, but on a dry watch crystal instead of under water. Since the body tissues other than fat body showed slight autolysis, in these experiments only the intestines were removed, the blood was left in, and the other tissues were strained out through four thicknesses of fine cheese-cloth, after shaking with solution. Eleven larvæ yielded approximately the same total N by this technique as ten pupæ, the pupal fat body tissues being more easily freed from the supporting structures, and these were the numbers utilized. One set of larval and pupal tissues was diluted 7 to 1 with a buffer mixture obtained by titrating 2 per cent phosphoric acid with powdered calcium carbonate to a pH of 6.8 (the normal pH of larval blood), with phenol red as indicator. This solution contained an excess of precipitated salts, and maintained a fairly constant indicator color in the autolytic mixture for 2 weeks. Another similar set of larval and pupal tissues was diluted with a buffer mixture of 2.5 per cent glacial acetic acid in water, titrated to a pH of 5.0 with powdered calcium carbonate. These mixtures were so acid that increase of acid due to autolysis would be insignificant. Toluene was added to excess. Samples of 1 cc. were taken at intervals, the first one about 20 minutes after the beginning of dissection, which was the time occupied by preparation of the samples. On these four samples (one larval and one pupal tissue each, of autolyses at 6.8 and 5.0), total nitrogens and non-protein nitrogens were determined by the micro method of Folin and Wu (1919) for blood. One-eighth of each 1 cc. sample diluted was used for total N, and on 2 cc. fractions of the 10 cc. tungstate filtrates from the remaining seven-eighths, duplicate non-protein N determinations were made, a total of twelve determinations for each

set of tests. With larger amounts of nitrogen per sample than the above the salts of the buffers used clouded the solutions, and the fat present caused considerable foaming, while the prolonged boiling, which was necessary, produced a heavy deposit of silicates in the digestion tubes. The solutions were nesslerized directly without distilling, in volumetric flasks, centrifuged, and read against a known standard in a Bausch and Lomb colorimeter. Success with this technique was only attained by using very small fractions of the autolysate sample for digestion. Duplicates ran usually within 5 per cent of each other, and the average was taken, unless a wider discrepancy was shown, when a third sample was run. Points plotted are for values of non-protein nitrogen over total nitrogen, per cc. of solution.

*Data.*—Curves of autolysis at controlled pH are given in Fig. 2. Ordinates are  $\frac{\text{Non-protein N}}{\text{Total N}}$  per 1 cc. sample of autolysate, abscissæ time in days. The total N per cc. was almost exactly 1 mg., or 8 mg. in the blood and fat body from eleven larvæ or ten pupæ. The ordinates represent, therefore, approximately the non-protein N per cc. of solution. The curve for larval tissue at pH 6.8 is represented by circles, for the corresponding pupal tissue by crosses, for larvæ at pH 5.0 by erect crosses, and for pupæ at 5.0 by triangles. The third set of readings appears to be uniformly too low, though the duplicate samples checked satisfactorily. This may have been the result of a slight cloud in the standard, and the discrepancy was not detected at the time. In the more alkaline samples the fat separated out and stuck to the side of the flask, occasioning some doubt as to the exact amount of N in the samples. The same error should be present in both sets, and is much less than the difference between these curves and the acid ones. In the latter, the fat appeared to be hydrolyzed to fatty acid, and while it separated out, it did not stick to the side of the flask.

It is evident that the curves run approximately parallel, those for the more acid autolysis running higher from the start than the others. The difference between the autolysis of larval and pupal tissues at pH 5.0 is well within the experimental error, and the curves may be considered identical. The autolyses at pH 6.8 start identically, but the larval tissues after the 3rd day

appear to fall below the pupal at this reaction by a value greater than a reasonable experimental error. There may have been a difference of a few tenths of one pH between these mixtures, for the finer shades of color from the indicator were obscured by the solids in the autolysate. The difference in autolysis here is considerably less than that generally observed in the former series of unbuffered mixtures, but it is in the same direction. Considerable excess of carbonate was present at pH 6.8, and unequal loss of  $\text{CO}_2$  may have caused this difference. The con-

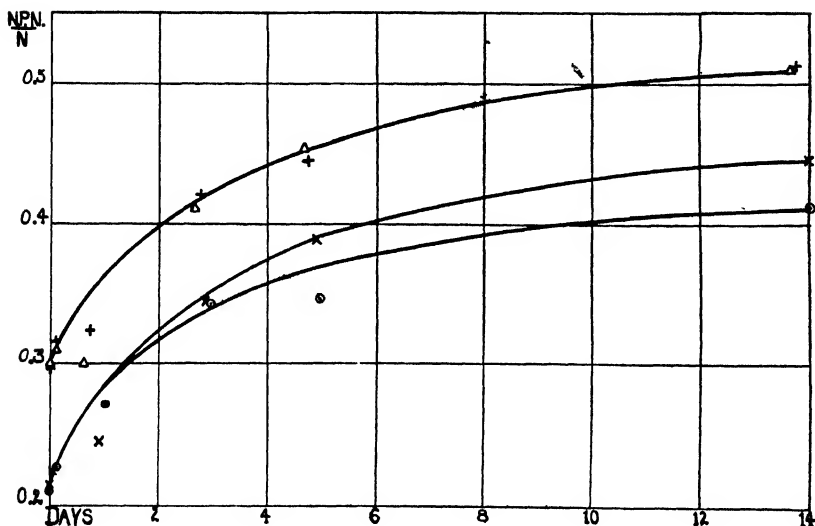


FIG. 2 Autolyses of larval and pupal tissues at controlled pH ○ represents larval tissue at pH 6.8, x, pupal tissue at pH 6.8, +, larval tissue at pH 5.0, and Δ, pupal tissue at pH 5.0. Further explanation in text

dition of the fat in these mixtures also caused difficulty in obtaining fair samples for analysis, as mentioned elsewhere. This shows in greater irregularity of the curves at pH 6.8 than at 5.0.

The most significant feature of these experiments is the initial rise in the non-protein N of the acid autolyses, which took place during the first few minutes after adding the buffer mixtures to the tissues, before the samples could be precipitated. This is undoubtedly due to acidity, since it occurs in both larval and pupal tissues, and in both sets of autolyses these tissues differ



from one another but slightly at the start. All the curves may, therefore, be considered to have risen from a common level, almost perpendicularly, for the first few minutes. The final levels of the curves are for the more alkaline autolyses about twice, for the more acid about two and a half times the initial non-protein N values, and represent autolysis of 40 and 50 per cent, respectively, of the total protein present, assuming practically all the nitrogen to be in the form of protein and its chemical fragments. Any purine derivatives present are further assumed to be in the same amounts in larvæ and pupæ, and to be present in relatively insignificant amounts.

*Discussion.*—These latter experiments afford an interpretation of the former series, and indirectly, of the processes occurring in the living organism. The following conclusions seem justifiable.

First, the differences in the rate and extent of autolysis of tissue from different stages, *in vitro*, are due to differences in acidity of the autolysate. When tissues are diluted with pure water, the buffering action of the mixture is lowered, and a slight excess of acidity in one tissue or the other would accelerate its rate of autolysis. Since at the same conditions of total acid and pH the two stages autolyze practically identically, with the same initial and subsequent amounts of amino nitrogen, a difference of rate in two non-buffered autolysates is presumptive evidence at least of a difference in effective acidity in the tissues to begin with.

Second, there is no appreciable difference in either the total protein present in the tissue of these different stages, nor in the fraction of the total nitrogen existing as amino-acids. The fact that eleven larvæ are required to furnish the same amount of total nitrogen as ten pupæ may be assigned to the circumstance that in the larvæ the cells of the fat body adhere more tenaciously to the body tissues that are strained out, as pointed out above (see foot-note to Table I). At the pupal stage investigated no great growth of pupal tissues had taken place to absorb material from the larval histolysis.

Third, the greater acidity that appears to be present in pupal tissues is not due to amino-acids. These would not affect the reaction materially anyway, except to determine possibly an

equilibrium end-point with the unhydrolyzed proteins, etc., at a given reaction. The acidity affecting autolysis in non-buffered solutions results from metabolic activity of the organisms before making up the solutions. As has been shown elsewhere (Bishop, 1923), the blood of pupæ has a lower alkali reserve than that of larvæ, though the pH is about the same, due to lower CO<sub>2</sub> values in the pupæ. If acid were liberated (such as lactic or phosphoric) from activity during cocoon spinning, this would affect unbuffered solutions of tissue materially, while the CO<sub>2</sub> would tend to pass off during autolysis, leaving the pupal tissues, with little CO<sub>2</sub> and much other acid, more acid than the larval, with much CO<sub>2</sub> and little other acid. Titration of tissue mixtures to neutrality preparatory to the Sorensen titration of amino-acids after adding formaldehyde indicated that the pupal tissue autolysates did produce more titratable non-amino-acids than the larval.

Finally, the abrupt and extensive initial rise in non-protein N in the acid autolysates may be assigned to the presence of a large amount of free acid which can combine with proteins at the existing pH to furnish a large initial amount of substratum for the action of autolytic enzymes. The autolysis of proteins (Bradley, 1922, *a*) takes place by two stages, the first consisting of the hydrolysis of the protein molecule in an acid medium at an optimum pH of 4 to 5, by one enzyme, the "primary tissue protease," followed by further splitting by another enzyme, ereptase, into amino-acids. The second part of the process takes place readily at either acid or alkaline reaction.

Further (Bradley, 1922, *b*), the H ion concentration determines the rate of the initial cleavage, but the total amount of amino-acids formed, *i.e.* the final level of the curve, will be determined by the total amount of acid present which can combine with the proteins at a given reaction. If this first concept is correct, since in all these solutions there must have been an excess of total acid, the lower level of the curves of the less acid autolysates must also have been due to the difference in H ion concentration, seemingly contrary to the second concept. This might be explained on the assumption either that the end-point of the final equilibrium between hydrolyzed proteoses, etc., and amino-acids, depended on the reaction, which is improbable, since the ereptase should dispose of protein fragments at either

reaction; or else that certain of the proteins were not hydrolyzed at all at the less acid reaction, being still on the alkaline side of their isoelectric points, and not, therefore, available as substrate for autolysis. If the latter is the case, the question arises: How does the living organism dispose entirely of the products of cellular disintegration at this pH? But this is perhaps applying the autolysis theory more literally to vital phenomena than the present knowledge of either warrants.

Once the initial rise is attained, the autolyses at pH 5.0 and 6.8, for both larvæ and pupæ, go on at virtually the same rate, giving parallel curves. There seems to be no present reason for assigning differences in proteolytic enzyme content of the larval and pupal tissues as a cause for the differences in autolysis.

Further light may be thrown on this process by consideration of the substances precipitated from these solutions by tungstic acid. Huller and Van Slyke (1922) have shown that the tungstate filtrate from blood contains practically all the amino-acids present, but that Witte peptones are largely thrown down. Assuming that the products of "primary tissue protease" hydrolysis are precipitated with the proteins, the autolysis figures give a measure directly of the splitting of these fragments to amino-acids by the ereptase present, but this is, of course, conditioned by the amount of hydrolyzed proteins produced by the primary enzyme. Therefore, since ereptase works at the reaction of either set of experiments, the difference in results due to the reaction may be assigned primarily to the primary hydrolysis. This would indicate that the regulating mechanism of autolysis is here the reaction controlling the primary cleavage, and one may infer that the equilibrium between protein and its first cleavage products is the point at which autolyses of the proteins is regulated both *in vitro* and *in vivo*; an hypothesis in accord with Bradley's statement. The sudden rise in the curves at the more acid reaction, and the subsequent nearly parallel course of all the curves, might be taken to indicate that the primary cleavage is a very rapid process in these tissues, while the secondary amino-acid production proceeds more slowly to an equilibrium level less dependent on the reaction. That is, each curve may really be resolved into two curves, the character of the first part of

the summed effect being determined chiefly by the primary hydrolysis of such proteins as will hydrolyze at the given reaction, and that of the latter part being determined chiefly by the rate of amino-acid production from the split protein products.

It may be inferred from these experiments that in the bee larva at commencement of pupation protein autolysis follows the increased acid content of the tissues, which is due chiefly to enclosure in a cocoon and the activity of spinning. Acidity may not be the only causative factor. The elaborate and orderly cytological changes taking place, which are not merely degenerative changes, may be connected with the autolytic process, but apparently do not alter the status of the proteolytic enzymes present. The nuclear granules that develop into the so called "albuminoid globules" in the cytoplasm, in particular, are probably concerned with fat metabolism, rather than with protein hydrolysis as generally assumed, especially since at this stage unsaturation of fat may be demonstrated in them by treatment with osmium tetroxide (Bishop, 1922). The degree and rate of autolysis that takes place in the living organism may be looked upon as resulting from a balance between total acidity, ferment action at the existing reaction, and utilization by growing tissues or other metabolic disposal of the end-products. Just why the reaction is maintained constant instead of running to the acid side as in autolysis *in vitro* it is impossible to state, but apparently the acids produced are disposed of as fast as formed, thus holding back the autolytic process to the rate demanded by the needs of the animal.

#### *Lipolysis in Protein Autolysis Mixtures.*

An attempt was made, on the first series of unbuffered autolyses, to determine the activity of lipase under these autolytic conditions. 1 cc. samples of the solutions were dried on filter paper over  $\text{CaCl}_2$  to constant weight, extracted with petroleum ether, and the extract was titrated to first change of color of phenolphthalein with 0.01 N alcoholic NaOH. The end-point was not sharp, due to gradual saponification of the fat by the alkali added. There were probably other acids such as hydroxy acids present. The fats separated out of the emulsion and collected on the sides of the flasks, making uniform sampling diffi-

cult. While a technique was finally developed, giving results on duplicate samples of known acid within 5 per cent on amounts as small as those in the samples utilized, the data on the actual determinations of fatty acid content were very erratic, and the method is not accurate enough to give satisfactory results. No differences could be determined by this technique between fatty acid produced in autolysates of larval and pupal tissues, though there was an increase demonstrable in the fatty acid titration in both as autolysis proceeded, but less in proportion to the total initial fatty acid than the concomitant increase in amino-acid. As large a difference as that which occurred in the protein autolyses would have been detected by this technique, unsatisfactory as it was. Further, the pupal tissue contained the same amounts of total ether extract as the larval, per gm. of dry weight of tissue, within the limits of experimental error of this series of experiments. This indicates that the disappearance of fats from globular form in the cytoplasm of the cells at the beginning of pupation may be a physical rather than a chemical process. Again, however, it should be emphasized that the later stage of pupal tissue utilized did not show complete disintegration of the fat body cells, but only an advanced stage of intracellular reorganization, and that the pupal tissue had not at this stage grown to any significant bulk to use up these materials.

When the results of fat autolysis were found not to be conclusive, the less specific test of the reaction of lipolytic enzymes on ethyl butyrate was made. The same stages of larval and pupal tissues were employed as above. Weighed amounts of the tissues were diluted to a common factor of wet weight, and each solution was divided into six equal parts, to four of which were added equal amounts of freshly neutralized ethyl butyrate. One sample containing ethyl butyrate and one blank were titrated immediately; the others after 15, 15, and 30 minute intervals, in an incubator at 40°C., and the second blank with the last. The reaction had reached a maximum at the end of an hour.

The results of these experiments show a positive action of tissue enzymes on ethyl butyrate, but the ester-splitting action of the different stages of tissue was not appreciably different. Assuming that the lipolytic enzymes present in the tissues affect ethyl butyrate in the same degree that they do the native fats

and lipoids of the tissue, a change in the effectiveness of the lipases of the cell, or in the end-point of their reaction, from the larval stage to the pupal, has not been demonstrated.

It appears from these experiments that the cell changes in the fat body during pupation do not result primarily in an increase of fatty acid due to lipase, though the latter is present in both stages. This does not mean that lipase is not active in the cells in removing the fat from the cytoplasmic fat globules. In whatever manner the fat is finally metabolized, it is probably first hydrolyzed and saponified. Oxidation of fats has been found to involve, in the forms studied, the so called unsaturation of the fatty acid with the subsequent oxidation of the two terminal C atoms to  $\text{CO}_2$ , and the formation of a molecule of fatty acid with two less C atoms in the chain. This may be considered tentatively to be taking place in these cells, the function of the lipase being then to maintain a supply of fatty acid hydrolyzed from globular fat. The so called "albuminoid globules" developing in these cells, whose development in the bee is initiated by nuclear material, and to whose subsequent enlargement all the materials of the cytoplasm contribute, must be the locus of this oxidative process if it is going on in these cells at all. A difference in the physical and chemical constants of the fat present in the two stages, larval and pupal, might be expected as a result. This aspect of the cell metabolism is being further investigated.

I am indebted to Dr. H. C. Bradley of the University of Wisconsin School of Medicine for suggestions as to the conduct of this work on autolysis.

#### SUMMARY.

This paper consists of an attempt to analyze the histolytic changes of metamorphosis in the bee larva *in vivo* by means of autolytic digestion experiments on certain of its tissues *in vitro*.

1. In unbuffered dilute solutions, the larval and pupal fat body tissues autolyze readily, the pupal in general to a greater degree than the larval. Other tissues autolyze distinctly less.

2. When the autolysis mixtures are buffered, the degree of protein autolysis depends on the pH of the autolysate of both larval and pupal tissues. The more acid mixtures autolyze

more completely, but the time to maximum is about the same as in the less acid mixtures.

3. In unbuffered mixtures, loss of  $\text{CO}_2$  may be a variable factor which, by allowing acidity to vary, causes differences in pH. In buffered solutions, at optimum autolytic pH, no evidence is obtained of differences in the activity of proteolytic enzymes in larval and pupal tissues.

4. Differences in acidity apparently affect the initial cleavage of proteins in these autolyses, and this is the point at which histolysis of proteins may be regulated in the living organism, since the pH and acid content have been shown to vary with conditions of metamorphosis in accordance with such an hypothesis.

5. Hydrolysis of ethyl butyrate by tissue enzymes gives no evidence of differences in the lipase activity of larval and pupal tissues.

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## THE INFLUENCE OF DIET ON TEETH AND BONES.\*

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### *Influence of a Scorbutic Diet on Teeth.*

It is well known from previous work that the antiscorbutic vitamin seems to be concerned in the calcium deposition in the organism. We know from the fundamental experiments on scurvy by Holst and Frolich (1) that, in guinea pigs fed on a scorbutic diet, marked changes occurred in the whole bony system with great fragility of the long bones. A faulty calcium metabolism must evidently be the underlying cause. These excellent experiments performed about 17 years ago have been verified by other investigators. As a result of these experiments several other investigators became interested in the question: Whether or not a lack or insufficiency of the antiscorbutic vitamin in the diet would have any influence on the formation and the maintenance of the teeth. In other words, whether or not the dental caries had any relation to the antiscorbutic vitamin. Dental caries has until recently been considered more or less a result of local processes in the mouth, the general metabolism not being regarded as playing any important part in the process of tooth decay. The earliest authors have looked upon the tooth as a fixed part of the body undergoing no change during different pathological processes in the general organism. In other words, the tooth has been regarded more or less as a dead substance.

There is, however, no reason to believe that the tooth should be any exception from other parts of the organism where an active metabolism is going on. Among the authors who have studied

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this question are Zilva and Wells (2), Robb and collaborators (3), and particularly Howe (4). They have all studied the influence of the antiscorbutic vitamin on teeth. The two former have found definite histological changes in the pulp and dentin from animals on a diet deficient in the antiscorbutic vitamin. Dr. Howe has investigated this question very thoroughly with experiments both on guinea pigs and monkeys, and has found definite macroscopical changes in the teeth, which seem to be identical with the human dental caries.

During 1921 and 1922 I had the opportunity to carry out some histological studies on the teeth from these guinea pigs, fed on a scorbutic diet. These studies showed plainly that histological changes had taken place on such a diet mainly in the same way as described by Zilva and Wells and Robb. The normal orthodentin was largely substituted by osteodentin. Such dentin is normally found closing the pulp chamber towards the cutting edge as the tooth is worn (guinea pig teeth have a persistent pulp and thus are constantly growing), but in a far less amount than in the teeth from scorbutic guinea pigs. In these guinea pigs the osteodentin—incorporating degenerated odontoblasts—reaches far down toward the apex of the tooth, and there is only a very narrow space of orthodentin surrounding it. The pulp tissue is so degenerated that one can scarcely recognize any of its normal elements. In the more severe cases there is nothing at all left of the proper pulp tissue, only some degenerated odontoblasts which might be seen scattered around. In many cases there seems to have been a fatty degeneration.

The process begins with hemorrhages in the upper part of the pulp and it extends down towards the apex.

The same process of degeneration in more or less degree, according to the length of time the animal has been on the diet, has been found in all the 200 sections made. The dentin seems to be more affected than the enamel. No sections have yet been made of the carious molars.

The salivary glands from guinea pigs (and from two monkeys), fed on the scorbutic diet, have also been studied, but as yet in only a few cases, and definite conclusions, therefore, cannot be drawn as to the difference in the glands between normal and scorbutic animals. It is safe, however, to say that some degenerative proc-

esses are going on. The glands are very much atrophied in size and the nuclei in the cells are partly destroyed. But not to such a degree as McCarrison (5) has found in other glands from scorbutic-fed guinea pigs and monkeys. (McCarrison did not make sections of the salivary glands, but compared the weights and found them to have decreased.)

If the tooth forms a living changeable part of the body as other organs do, contrary to the earlier idea, we should expect to find chemical changes going on in the tooth during metabolism disturbances, as for instance in scurvy. Bahrtdt and Edelstein (6) found marked changes in the bones of scorbutic infants; that is, a marked decrease in the total ash, calcium, and phosphorus content. Chemical analyses of teeth from scurvy have not yet been performed, so far as I know.

My chemical analyses have been performed on front teeth from fifteen scorbutic guinea pigs and from four normal guinea pigs (fed, three on a mixed diet, and one on a control diet).

The scorbutic diet was as follows:

Soy bean	50
Rolled oats to	100
Dried whole milk	10
Yeast	4
Butter	5
Agar	3
Calcium carbonate	1 5
Sodium chloride	1

The control diet used was the above mentioned diet with added orange juice.

In some of the teeth only calcium has been determined, in others calcium, magnesium, and total ash. Just the visible part of the front tooth was taken and dried for 20 hours at 100°C., cooled in a desiccator, and weighed. Then the tooth was ashed in a platinum crucible, brought to a constant weight, and the ash recorded. The ash was dissolved in approximately 0.5 N HCl and calcium and magnesium were estimated according to the method of Tisdall and Kramer (7). Table I represents the amount of ash, CaO, and Mg of front teeth from normal guinea pigs. Table II represents the findings from scorbutic guinea pigs. Table III represents the average figures from Tables I and II. This table shows that a

reduction in the amount of ash and CaO, and an increase in the amount of magnesium, have been found in the teeth from scorbutic animals compared with normal animals.

The reduction in ash and calcium is not so pronounced as we would expect to find it compared with the histological findings mentioned and the macroscopical picture. This must be due to the fact that the pulp chamber is partly filled up by a pathological calcification, as the sections show. Such pathological deposits of calcium are often found following a degeneration of organic matter, and in the scorbutic guinea pigs and monkeys there have been found such deposits in many places in the body in nearly every case.

The reduction in ash and calcium and the increase in magnesium are greatest where the diet has been deficient in calcium as

TABLE I  
*Normal Guinea Pigs.*

Guinea pig No	Diet	Ash of dry tooth	CaO of dry tooth	CaO of ash	Mg of dry tooth	Mg of ash
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
a	Mixed food	Ash not recorded	38 10		1 41	
b	Control	" " "	40 04		1 09	
c	Mixed food.	78 61	38 38	48 82	1 57	2 00
d	" "	78 55	38 20	48 63	1 16	1 48
Average		78 58	38 68	48 72	1 31	1 74

well as in vitamin C. As we see the magnesium content has increased to more than double in scorbutic guinea pigs, and as a rule a decrease in calcium content corresponds to an increase in the magnesium content.

This substitution of calcium by magnesium must be regarded as Nature's attempt to keep up the amount of salts in the teeth when she is in need of or unable to utilize the calcium. But it is a pathological process and must in this respect be classified under the term osteomalacia, where there usually is found an increase in magnesium and a decrease in the calcium of the bones (Hammarsten (8)). Besides the change in the organic matter the high magnesium content may account for the very brittle condition of the teeth in scorbutic-fed guinea pigs.

Thus we see that a diet deficient in the antiscorbutic vitamin is able to produce both histological and chemical changes in guinea pig teeth. We have, however, to remember that a guinea pig

TABLE II

Guinea Pig No	Diet	Ash of dry tooth	CaO of dry tooth	CaO of ash	Mg of dry tooth	Mg of ash
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
10	Scorbutic.	Ash not recorded	33 44		Mg not estimated	
15	"	" "	33 30		" "	
18	"	" "	37 08		" "	
14	"	" "	35 11		" "	
2	"	" "	35 80		2 86	
17	"	" "	35 45		2 95	
35	"	" "	36 15		2 15	
33	"	" "	36 63		2 24	
16	"	77 09	37 74	48 95	2 43	3 81
19	"	77 25	35 41	45 83	2 48	3 21
15	"	77 35	35 95	45 79	2 32	2 99
23	" plus Ca-deficient.	73 05	34 36	47 18	2 86	3 91
24	" " "	63 81	33 93	53 11	3 96	5 14
25	" " "	69 79	30 22	43 30	4 53	6 50
44	Scorbutic, some orange juice	77 98	36 39	46 38	1 80	2 30
Average		73 76	35 06	47 08	2 78	3 98

TABLE III

	Ash of dry tooth	CaO of dry tooth	CaO of ash	Mg of dry tooth.	Mg of ash
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Average of normal	78 58	38 68	48 72	1 31	1 74
" " scorbutic	73 76	35 06	47 08	2 78	3 98
Reduction from normal, <i>per cent</i>	6 00	9 00	3 4		
Increase above normal, <i>per cent</i>				112 00	130 00

tooth is a constantly growing one and it may be that the organism, when fed on a diet deficient in the antiscorbutic vitamin, is not able to form a tooth of normal composition. In this way our

original question may still be open for discussion: Whether or not an already formed tooth can undergo changes during a faulty diet and thus predispose to tooth decay. Animals with not constantly growing teeth have to be studied in order to settle this very important question. The best animals for this purpose would, of course, be monkeys and as mentioned above Dr. Howe has been able to produce caries-like defects in their teeth on a scorbutic diet. Monkeys are, however, expensive animals and are difficult to keep under laboratory conditions.

*Influence of a Calcium-Deficient Diet on Teeth and Bones during Pregnancy.*

As we have seen earlier in this paper, the macroscopical defects and the histological and the chemical changes in the teeth were most pronounced when the diet was deficient both in antiscorbutic vitamin and in calcium. There is a period in the mammal's life when the organism is in great need of calcium. This is during pregnancy. During this time we know that the calcium in the food must cover not only the requirements of the mother's own organism but also that of the growing fetus. An animal on an unrestricted diet will instinctively take the food needed whereas the adult human being, who in a certain way has lost her instinct very often during this period, is in lack of several food elements particularly some of the salts, such as calcium. Sherman (9) says that the diet is probably more deficient in calcium than in any other food element. The diet should contain at least 0.67 gm. of calcium per day per man (70 kilos). He has found that 50 per cent of American diets contain less than this.

It is a well known fact that the pregnancy period for many women is a very harmful one as far as the teeth are concerned. The old saying, "One tooth for each child," is certainly true even now for a large class of women both in America and Europe. The question is, to what is this due?

In accordance with earlier authors, this extreme tooth decay may be due partly to lack of oral hygiene, partly to a change in the hydrogen ion concentration of the blood towards the acid side (Peterson (10) and others).

It seems quite reasonable to assume that the rapid disintegration of the teeth during pregnancy is a consequence of the more

or less faulty diet during this period. The changes in the teeth which Hoffstrom (11) has characterized as physiological seem rather to point in the direction of direct pathological processes going on in the mother's organism caused by an insufficient diet. If an insufficiency is present in the food during pregnancy, the growing fetus is trying to take what it needs, while the old organism will suffer first.

In order to ventilate the two following questions some experiments on pregnant rats were started. (1) Is it possible to produce chemical changes in an already formed outgrown tooth by metabolic disturbances caused by a change in the diet? (2) Is the destruction of teeth during pregnancy due to a faulty diet with special reference to a calcium deficiency?

The white rat was chosen as the experimental animal as its molar teeth have a closed pulp chamber, and thus do not grow after their formation. The teeth mentioned come in every respect very near to the human molars. The rat is, furthermore, a rapidly growing animal, and its pregnancy period is only 21 days. The diet these rats were given was that of Osborne and Mendel (12) and consisted of: Whole wheat 92 per cent, butter 5 per cent, and salt mixture, except for the calcium, 3 per cent.<sup>1</sup> Six female rats and two male rats were kept on this diet. Two female rats were kept on a control diet, which was the same as above only with the addition of the calcium to the salt mixture (0.66 per cent  $\text{CaCO}_3$ ).

<sup>1</sup> Salt mixture:

	gm
$\text{CaCO}_3$	134 8
$\text{MgCO}_3$	24 2
$\text{Na}_2\text{CO}_3$	34 2
$\text{K}_2\text{CO}_3$	141 3
$\text{H}_3\text{PO}_4$	103 2
$\text{HCl}$	53 4
$\text{H}_2\text{SO}_4$	9 2
Citric acid plus $\text{H}_2\text{O}$	111 1
$\text{Fe citrate} + 1\frac{1}{2} \text{H}_2\text{O}$	6 34
$\text{KI}$	0 020
$\text{MnSO}_4$	0 079
$\text{NaF}$	0 248
$\text{K}_2\text{Al}_2(\text{SO}_4)_4$	0 0245

The whole wheat was ground in the laboratory mill and the other constituents were mixed together. The food needed per day was made up to a paste with distilled water. Distilled water was given the rats to drink.

It very soon became evident that the animals kept on this calcium-deficient diet were not able to breed normally. One female rat did not get pregnant at all during the experimental period of 62 days. Four became pregnant once, and only one became pregnant twice. It evidently was the low calcium content of the diet, which was the reason for the very poor breeding, as the control animals went through a normal number of pregnancies during the same period. It was, furthermore, very difficult to raise the young as they very often were killed and eaten by the mother. Whether this was due to a calcium hunger or to a low milk secretion of the mother is difficult to tell. In accordance with the work of Evans and Bishop (13) raw potatoes were given to two of the females for the last month in order to add to their diet the possible specific vitamin for breeding. No effect was noticed, however.

In order to study the calcium metabolism on such a calcium-deficient diet four metabolism studies were undertaken—two on pregnant rats and one on a non-pregnant rat, all three animals being kept on a calcium-deficient diet. One study was performed on a pregnant rat on a control diet. The metabolism period was from 3 to 4 days. A special metabolism cage for rats was used and the urine and feces were collected on salt-free filter paper. For calcium, magnesium, and phosphorus the exact intake and output were determined. The method used for calcium was that of McCrudden (14), modified according to Shohl (15), Simpson (16), and Briggs (17). For magnesium and phosphorus the methods of Briggs (17) were used.

The results of these metabolism studies appear in Table IV.

It is seen from Table IV that Rat 7 (the pregnant control rat) has retained, calculated per gram of body weight, nearly three times as much calcium as the pregnant rat, No. 4, which was kept on a calcium-deficient diet. The control rat, however, retained much less magnesium (one-fifth) than Rat 4. The organism tries evidently to substitute the lacking calcium with magnesium. The control rat retained a little more phosphorus than Rat 4.

TABLE IV  
*Metabolism Studies.*

Rat No.	Condition.	Initial weight	Loss or gain in weight.	Experimental days	Average total food intake in 24 hrs	Constituents examined	Average intake in 24 hrs	Average excreted in urine in 24 hrs	Average excreted in feces in 24 hrs	Balance	Absorption	Retention	
												per cent	per gm. of middle body weight.
4	Pregnant.	154	+35	3	11.7	Ca Mg P	5.6 33.2 57.0	17.4 10.7 9.9	1.07 16.6 8.8	+2.79 +5.90 +38.3	81.0 50.0 84.6	50.0 17.8 67.0	0.162 0.343 0.223
4	Not pregnant.	131	+10	3	5.9	Ca Mg P	2.3 14.7 39.5	1.3 3.9 14.0	1.25 10.7 10.2	+0.25 +0.1 +15.3	55.5 92.7 74.2	+ 0.68 38.7	+ 0.0007 0.113
3	Not pregnant.	115	+2	4	4.9	Ca Mg P	1.9 12.0 31.0	0.53 2.64 11.7	1.19 7.75 7.25	+0.18 +1.61 +12.05	37.5 35.5 76.5	9.0 13.4 38.7	0.00155 0.0139 0.104
7	Pregnant control.	180	+5 Did not eat very well.	4	5.9	Ca Mg P	31.4 12.3 39.3	2.42 4.35 12.5	20.5 6.7 9.7	+8.48 +1.25 +17.1	35.5 55.5 75.5	27.0 10.2 43.5	0.0463 0.007 0.096



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The metabolism study performed on Rat 4 7 days after delivery of 11 young and 9 days after the first metabolism period was completed showed a negative calcium balance and a very low magnesium retention. This negative calcium balance may at this time be a result of the previous pregnancy. The pregnant rat had to use all the calcium in the diet for her fetus and nothing was left for her own organism. Some time may perhaps pass before the organism again gets used to depositing the calcium present in the diet. The other non-pregnant rat, No. 3, shows a very low, although positive, calcium balance.

TABLE V.  
*Inorganic Constituents of New-Born Rats.*

Rat	Ash of body weight	Ca of body weight	Ca of ash	P of body weight	P of ash	Mg of body weight	Mg of ash
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Young from normal rat	1 620	0 2770	17 10	0 260	16 40	0 0206	1 268
“ “ “ “	1 532	0 2640	17 22	0 274	17 36	0 0230	1 522
“ from experimental Rat 2 .	1 616	0 2475	15 30	0 248	15 40	0 0217	1 340
Young from experimental Rat 5	1 595	0 2800	17 32	0 260	16 20	0 0320	1 980
Young from experimental Rat 1, second pregnancy.	1 339	0 2360	17 60	0 232	17 35	Spoiled.	
Average of normal rats	1 576	0 2705	17 16	0 267	16 88	0 0218	1 395
“ “ treated “	1 516	0 2545	16 74	0 246	16 31	0 0268	1 660

By examining the new-born from the rats on the calcium-deficient diet a very small lowering was found in the total ash, calcium, and phosphorus while the magnesium had increased a little. This very small change which may be seen from Table V demonstrates that the old organism provides for the young as far as possible even when the mother has to take the different elements from her own body.

The chemical analyses of the bones and teeth of the rats fed on the calcium-deficient diet bring out marked differences from the composition of the bones and teeth of the control animals.

Table VI shows that the average figures for total ash, calcium, and phosphorus in the bones of the treated adult rats lie far

below those for the normal adult rat. The magnesium content, however, has definitely increased. The percentage of magnesium of the bone ash of the rats on the diet is increased about 50 per cent.

TABLE VI  
*Analyses of Bones (Diaphysis of Femur).*

Rat No.	Sex.	Condition.	Ash of dry bone	Ca of dry bone	Ca of ash.	P of dry bone	P of ash.	Mg of dry bone	Mg of ash.
			per cent	per cent	per cent	per cent	per cent	per cent	per cent
A	M.	Normal rat fed on mixed diet.	67 0	26 0	38 8	12 04	17 93	0 46	0 69
B	F.	“ “	65 9	24 7	37 6	11 93	17 93	0 51	0 76
C	M	“ “	68 8	27 2	41 0	11 5	17 5	0 54	0 79
1	F.	On the Ca-poor diet for 90 days, 2 pregnancies	55 0	20 9	37 8	9 6	17 4	0 47	0 85
2	“	On the Ca-poor diet for 81 days, 1 pregnancy	50 2	18 9	37 6	8 63	17 2	0 42	0 83
3	“	On the Ca-poor diet for 62 days, no pregnancies	52 8	20 3	38 7	9 75	18 5	0 7	1 33
4	“	On the Ca-poor diet for 77 days, 1 pregnancy.	53 9	20 2	37 5	9 3	17 3	0 59	1 10
5	“	On the Ca-poor diet for 73 days, 1 pregnancy	50 0	18 25	36 5	9 03	18 1	0 58	1 16
6	“	On the Ca-poor diet for 77 days, 1 pregnancy.	52 1	19 7	37 9	9 04	17 3	0 57	1 09
8	M.	On the Ca-poor diet for 61 days	57 5	21 7	37 8	7 6	13 2	0 5	0 86
9	“	On the Ca-poor diet for 44 days	62 4	24 0	38 5	7 9	12 7	0 57	0 91
Average of normal adult rats			67 5	26 0	39 0	11 9	17 8	0 51	0 75
“ “ treated “ “ . .			54 3	20 6	37 8	8 85	16 5	0 55	1 12

The chemical picture in these bones is thus similar to that found in osteomalacia as described by Hammarsten (8). Chabré (18) found such a high magnesium content in the bones from osteomalacia that the magnesium content was even higher than that of calcium. McCrudden (19) also found a higher magnesium content than calcium.

White (20) found a marked diminution in calcium with an excess of sulfur and magnesium. Several authors have examined the chemical composition of bones from animals on a calcium-deficient diet. Most of them agree that the calcium content is low. Very few have examined the magnesium content and, where it has been determined, it was mostly found low.

Weiske (21) found no change in the composition of bones from animals on a calcium- and phosphorus-deficient diet. In a later work he found no increased magnesium content in the bone. Beraz (22) found a lowered calcium percentage both in bones and soft tissue. A test was not made for magnesium. Perlzweig (23) has found a reduction in the total ash, calcium, phosphorus, and magnesium in the bones from rats fed on a calcium-deficient diet. Aron and Sebaue (24) showed that the bones from animals on a calcium-deficient diet are lower in dry substance, which has a lower ash content, whereas the calcium of the ash was hardly diminished.

Table VII shows the chemical picture of the analyses of the molar teeth of the same rats.

We see that the chemical changes in teeth have gone in just the same direction as those in the bones. The total ash, calcium, and phosphorus have all decreased, while the magnesium has increased.

Table VIII contains the analyses of front teeth from the same adult rats and gives us a picture of a different process.

As we see, the total ash, calcium, phosphorus, and magnesium have all decreased. The magnesium has here followed the other constituents, that is, the picture corresponds mostly to a rachitic process.

There are very few analyses of rachitic bony material in the literature, but the one which has been found shows the same process. Aron (25) has found that a rachitic child's femur contained less calcium and phosphorus, as well as magnesium, than that of a normal child.

Perlzweig (23) has, furthermore, also found a lowering in the total ash, calcium, phosphorus, and magnesium in the front teeth from rats fed on a calcium-deficient diet. Analyses of the molar teeth in rats have not been found in the literature.

When we compare Tables VII and VIII, we see that a different process is taking place in the molar and front teeth of the same

animal. While the magnesium in the front teeth is following the other constituents in a lowering, it is increasing in the molar teeth, while the others are decreasing.

We have to remember that the molar teeth were formed with a closed pulp chamber when the animals were started on the diet,

TABLE VII  
*Analyses of Molar Teeth*

Rat No	Sex.	Condition	Ash of dry tooth	Ca of dry tooth	Ca of ash	P of dry tooth	P of ash	Mg of dry tooth	Mg of ash
			per cent	per cent	per cent	per cent	per cent	per cent	per cent
A	M	Normal rat fed on mixed diet	78 0 31	1 41 0	14 2	18 4	0 35	0 48	
B	F	“ “	78 5 30	5 39 0	14 8	18 9	0 46	0 63	
C	M.	“ “	78 8 30	5 38 8	14 5	18 4	0 57	0 73	
1	F.	On the Ca-poor diet for 90 days, 2 pregnancies	76 5 30	0 39 2	14 4	18 9	0 71	0 92	
2	“	On the Ca-poor diet for 81 days, 1 pregnancy.	76 3 29	0 38 0	13 65	17 9	0 75	0 98	
3	“	On the Ca-poor diet for 62 days, no pregnancies	77 0 30	2 39 2	14 16	18 45	1 05	1 37	
4	“	On the Ca-poor diet for 77 days, 1 pregnancy	75 5 29	2 38 6	13 8	18 25	0 83	1 10	
5	“	On the Ca-poor diet for 73 days, 1 pregnancy	75 0 29	5 39 5	14 3	19 1	Not determined.		
6	“	On the Ca-poor diet for 77 days, 1 pregnancy	77 4 30	21 39 1	14 0	18 1	0 77	0 99	
8	M	On the Ca-poor diet for 61 days	77 5 30	3 39 0	13 48	17 3	0 62	0 80	
9	“	On the Ca-poor diet for 44 days	75 8 29	7 39 3	14 35	18 9	0 68	0 89	
Average of normal rats			78 4 30	7 39 6	14 5	18 6	0 46	0 61	
“ “ treated “			76 4 29	7 38 9	14 0	18 38	0 77	1 01	

whereas the front teeth were constantly growing during the experimental period. It is most natural to consider the process going on in the molar teeth as osteomalacia and that in the front teeth as rickets. Both processes are thus an expression of a calcium deficiency, except that the organism answers in a different

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way according to the state of the tissue, whether it is growing or not.

We see from these tables that the changes produced by the diet have taken place both in the bones and teeth of the same animals, and even in a fixed, formed tooth like that of a human being. The

TABLE VIII  
*Analyses of Front Teeth.*

Rat No	Sex	Condition	Ash of dry tooth	Ca of dry tooth	Ca of ash.	P of dry tooth	P of ash	Mg of dry tooth	Mg of ash.
			per cent	per cent	per cent	per cent	per cent	per cent	per cent
A	M	Normal rat fed on mixed diet.	78 2	28 95	37 1	14 75	18 86	1 52	1 94
B	F.	“ “	78 3	28 8	36 85	14 52	18 65	1 68	2 14
C	M.	“ “	79 8	29 0	36 5	15 7	19 7	1 8	2 27
1	F.	On the Ca-poor diet for 90 days, 2 pregnancies	74 3	25 2	33 9	13 6	18 2	0 83	1 12
2	“	On the Ca-poor diet for 81 days, 1 pregnancy.	75 4	28 2	37 4	13 3	17 7	0 68	0 91
3	“	On the Ca-poor diet for 62 days, no pregnancies	76 3	28 6	37 5	14 25	18 7	1 01	1 33
4	“	On the Ca-poor diet for 77 days, 1 pregnancy	76 5	28 0	36 6	13 5	17 7	0 96	1 25
5	“	On the Ca-poor diet for 73 days, 1 pregnancy	74 5	27 2	36 5	13 8	18 5	0 75	1 00
6	“	On the Ca-poor diet for 77 days, 1 pregnancy	71 0	26 8	37 9	13 05	18 4	0 79	1 16
8	M	On the Ca-poor diet for 61 days	77 6	28 15	36 25	13 44	17 3	0 90	1 16
Average of normal rats			78 5	28 9	36 8	14 9	19 1	1 67	2 12
“ “ treated “			75 0	27 4	36 6	13 5	18 1	0 85	1 13

changes in the bones are more far reaching than those in the teeth merely because the circulation in the bones is better than in the teeth and consequently the changes are more rapid in the former than in the latter.

Calcium in the blood has been determined on three normal and seven treated rats. While the figures for the normal rats lie

around 11 mg. per 100 cc. of blood serum the figures for the rats on the calcium-deficient diet lie far below.

As will be seen from Table IX the average figure for seven treated rats is 6.49 mg. per 100 cc. of serum. No convulsions occurred in any of the rats.

In the discussion whether or not we have carried our fundamental question as to dental caries any nearer to a solution, we have to take into account the resistance of the tooth. It seems quite natural that the resistance of the tooth against decay must lie in the structure and chemical composition of the tooth. Changes in these

TABLE IX.  
*Analyses of Calcium in Blood Serum.*

Rat No	Sex	Condition	Calcium per 100 cc. serum
			mg
C	M.	Normal rat fed on mixed diet.	11 1
D	"	" " " " " "	11 1
E	"	" " " " " "	12 0
2	F.	On Ca-poor diet for 81 days, 1 pregnancy.	5 0
4	"	" " " " 77 " 1 "	5 88
5	"	" " " " 73 " 1 "	6 80
6	"	" " " " 77 " 1 "	5 88
8	M.	" " " " 61 "	6 4
9	"	" " " " 44 "	8 1
14	"	" " " " 30 "	7.4
Average of normal rats			11 4
" " treated "			6 49

two factors may be the first step in the process of decay. These changes whether they are only of a chemical character or of a chemical and physical one may be quite small and yet quite sufficient to render the tooth susceptible for a third factor, which may be a local one, to enter in and make the final step in the process of decay.

Regarding the two questions put before us in this paper, the first question, namely "Is it possible to produce chemical changes in an already formed tooth by changing the diet," can be answered positively, based on the studies in this article.

The second question, "Is the destruction of teeth during pregnancy due to a faulty diet with a special reference to a calcium deficiency," is, however, still open for discussion. It was unfortunate that the animals in these series were kept on such a low calcium diet that they did not breed sufficient numbers to study the influence of pregnancy on the teeth. Several series of rats have now been started on a more abundant calcium diet in order to study the influence of this factor.

#### SUMMARY.

Histological and chemical studies have been made on front teeth from guinea pigs fed on a scorbutic diet (Dr. Howe's animals). These studies have shown that the histological picture is a quite different one from that of normal animals. The pulp is so degenerated that one can scarcely recognize any of its normal elements and the normal orthodentin is largely substituted by osteodentin. The chemical picture is also altered. A marked decrease in total ash and calcium oxide and a marked increase in magnesium have been found. (Phosphorus was not determined.)

White female rats were fed on a low calcium diet (no calcium added to a mixture of whole wheat, butter, and Mendel and Osborne's salt mixture minus calcium) in order to study the effect of pregnancy on the teeth during a period of calcium deficiency. The rats, however, did not breed sufficiently to study the factor of pregnancy (possibly an account of the low calcium content in the food).

Metabolism studies during the calcium-deficient period showed, compared with animals on a control diet, a low calcium retention with an abnormally high magnesium retention.

The blood calcium had fallen from between 11 and 12 mg. of calcium per 100 cc. of serum in normal rats to as low as 5 mg. in rats on the diet. No tetany was observed.

The bones from the treated rats showed uniformly a great reduction in total ash, calcium, and phosphorus with a possible increase in magnesium.

Chemical changes have occurred as a result of the low calcium diet both in the front teeth (constantly growing) and in the molar teeth (formed, not constantly growing). The chemical process in the two kinds of teeth seems to be a different one. Analyses of

the molars show a reduction in the total ash with a small decrease in the calcium and phosphorus and a small increase in the magnesium content. Analyses of front teeth show a reduction in all respects.

It is seen from these studies that it is possible to produce chemical changes in an already formed tooth by changing the diet.

It is a great pleasure for me to extend my sincere thanks to Dr. Percy R. Howe, The Forsyth Dental Infirmary for Children, Boston, for the opportunity of using his animals for the studies on guinea pigs, and for the help and constant encouragement during these studies.

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## THE ESTIMATION OF BILE ACIDS IN URINE.

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The condition in which the tissues of the body are stained with bile pigments is known as icterus or jaundice. On account of the characteristic pigmentation and the appearance of bile pigments in the blood and in the urine the disease was recognized at an early date. A great many factors apparently may lead to this condition; usually, however, it involves obstruction of the common bile duct. The appearance of pigments in the urine indicates that a channel for their elimination is afforded and investigators were early led to look for a similar excretion of the bile acids. The results of repeated investigations are not altogether in harmony, probably due in large part to the lack of accurate methods for the estimation of the bile acids. The general consensus of opinion is that the bile acids are eliminated in the urine only in small quantities and this at once raises the question as to the fate of these substances. It appears possible that either through liver injury or perhaps by means of a mechanism which controls the synthesis of bile acids that in obstruction of the bile duct there is a decreased production; it is not, however, conceivable to us that the synthesis of bile acids is wholly stopped. The failure of certain investigators to find bile acids in appreciable amounts in urine seemingly indicates that they are oxidized in the tissues; this explanation, however, cannot be unqualifiedly accepted, especially when it is recollected that taurine which is a constituent of taurocholic acid, on account of the sulfonic acid radical, is not oxidized to any appreciable extent (1).

In order that the entire subject may be more thoroughly studied it appeared to us that an accurate method for the quantitative estimation of the bile acids in urine is a necessary prerequisite.

The methods which have previously been employed for the estimation of bile acids in urine may conveniently be grouped into three classes: (a) Color tests (2, 3, 4); all of which are essentially modifications of the well known Pettenkofer test. This has repeatedly been shown to be not specific for the bile acids since a large variety of substances respond to the test. Various workers have attempted either by precipitation or by extraction to free the bile salts from the interfering substances before applying the Pettenkofer test, but at its best this method from the quantitative aspect is but an approximation. (b) Isolation of the bile acids (5). This is a difficult process especially when small quantities are present in a complex medium such as urine and a high degree of accuracy cannot be expected. (c) Methods which depend on the change of surface tension which is produced in urine by the presence of bile acids (6). This has been measured either by means of the stalagmometer or by placing flowers of sulfur on the surface of the urine which is to be tested for the presence of bile acids. If the latter are present the sulfur particles will either sediment or spread over the surface of the fluid. This is commonly known as Hay's test. The presence of protein and probably other substances in the urine interferes seriously with the estimation of the bile acids.

In 1919 Foster and Hooper (7) published a method for the estimation of bile acids in small quantities of dog bile. Advantage is taken of the fact that on hydrolysis with alkali taurocholic acid is split into cholic acid and taurine. The estimation of the increase of amino nitrogen as the result of the hydrolysis serves as the measure of the amount of taurine which is set free and hence the concentration of the bile acids in bile. This method has been further modified so that glycocholic and taurocholic acid may be individually estimated (8), when both are present in bile. The principle underlying the method of Foster and Hooper offers a possible means for the estimation of the bile acids in urine provided certain interfering substances, particularly proteins, amino-acids, hippuric acid, and urea, can be eliminated. In order to accomplish this we have taken advantage of an observation made by Tengström (9) who found that the bile acids may be precipitated when in pure solution by saturating the solution with  $\text{MgSO}_4$ . Experiments were carried out by us to check the findings

of Tengström and to determine the completeness of the precipitation of the bile acids by the addition of magnesium sulfate. An alcoholic solution of the bile acids from ox bile was prepared by adding 10 volumes of absolute alcohol to bile, heating to effect solution of the bile acids and to coagulate the proteins. The latter were removed by filtration and four samples of equal volume were evaporated to dryness. The residues were then treated as follows: One was dissolved in distilled water; the second was taken up in about 8 cc. of 8 per cent NaOH solution and the bile acids were hydrolyzed by placing the flask containing the solution in boiling water for 8 hours; the third was dissolved in 15 cc. of water and the solution was then saturated by the addition of solid  $\text{MgSO}_4$ . After standing in the ice chest overnight the residue was filtered off on a Gooch crucible, then washed with small quantities of a cold saturated solution of  $\text{MgSO}_4$ , and the bile acids were finally extracted with hot absolute alcohol. The alcoholic solution was evaporated to dryness and the residue was dissolved in water. The fourth sample was similarly treated except that after evaporation of the alcohol the residue was dissolved in 8 per cent NaOH and hydrolyzed in a manner similar to the second sample. All the final volumes were adjusted to 10 cc. and the estimations of amino nitrogen were carried out according to the well known method of Van Slyke.<sup>1</sup> 2 cc. of solution were used for each estimation. The difference for the N values which were obtained for the hydrolyzed and for the unhydrolyzed samples gives the nitrogen of the bile acids. The results which were obtained in this experiment are given in Table I and indicate a recovery of 97 per cent.

Experiments were next carried out to determine the recovery of the bile acids from urine. The urine to which an alcoholic solution of the bile acids had been added was evaporated to dryness, the bile acids were extracted with absolute alcohol, the alcohol was removed by evaporation, and the bile acids were precipitated from their aqueous solution (15 cc.) by the addition of solid  $\text{MgSO}_4$  to saturation. The remainder of the procedure

<sup>1</sup> In order to eliminate the possibility of slowly reacting amines being present, estimations were made by shaking the bile acid solution with  $\text{HNO}_2$  for 4 and 8 minutes, respectively. The nitrogen due to slowly reacting amines was found to be a very small quantity.

was the same as that which is given in the previous experiment with bile. A satisfactory recovery of the bile acids is indicated in the results which are given in Tables II and III.

TABLE I  
*Estimation of Bile Acids in Ox Bile.\**

	mg.
Method of Schmidt and Dart.	
Amino nitrogen after hydrolysis	3 8
“ “ before “ . . . .	0 6
Nitrogen of bile acids . . . .	3 2
Precipitation by magnesium sulfate	
Amino nitrogen after hydrolysis	3 7
“ “ before “ . . . .	0 6
Nitrogen of bile acids . . . .	3 1
Percentage recovery of bile acid nitrogen	97

\* The figures which are given in Tables I, II, III, and IV for the amino nitrogen before and after hydrolysis include the reagent blank. Since the bile acid nitrogen represents the increase of amino nitrogen due to the hydrolysis of the bile acids it is unnecessary to determine the blank on the reagents.

TABLE II  
*Recovery of Taurocholic Acid and of Glycocholic Acid After the Addition of These Substances.*

	Taurocholic acid	Glycocholic acid.
Estimation of bile acid nitrogen in the aqueous solution.		
	mg.	mg
Method of Schmidt and Dart		
Amino nitrogen after hydrolysis	1 47	1 65
“ “ before “ . . . .	0 38	0 08
Nitrogen of bile acid . . . .	1 09	1 57
Estimation of bile acid in urine.		
Precipitation by magnesium sulfate.	*	†
Amino nitrogen after hydrolysis . . . .	1 58	2 60
“ “ before “ . . . .	0 40	0 99
Nitrogen of bile acid	1 18	1 61
Percentage recovery of bile acid nitrogen from urine . .	108	102

\* 40 mg. of taurocholic acid per 200 cc. of urine.

† 52 mg. of glycocholic acid per 200 cc. of urine.

Another series of experiments was carried out in which both whole human and ox bile in varying amounts were added to specimens of urine. The analytical procedures were the same as those which were employed in the previous experiment. The results which are given in Table IV indicate a recovery greater than 90 per cent of the bile acids from urine in each instance.

An experiment was next carried out to determine whether the presence of hippuric acid interferes with the estimation of the bile acids in urine. 96 mg. of hippuric acid were added to 50 cc. of urine and the resulting solution was treated in a manner similar

TABLE III

*Recovery of Bile Acids from Urine After the Addition of an Alcoholic Solution of the Bile Acids from Ox Bile.*

	Specimen 1	Specimen 2.
Estimation of bile acid nitrogen in the alcoholic solution of ox bile.		
	mg.	mg.
Method of Schmidt and Dart.		
Amino nitrogen after hydrolysis	3 8	4 8
“ “ before “	0 6	0 7
Nitrogen of bile acids	3 2	4 1
Estimation of bile acids in urine.		
Precipitation by magnesium sulfate		
Amino nitrogen after hydrolysis	3 8	4 7
“ “ before “	0 7	0 7
Nitrogen of bile acids	3 1	4 0
Percentage recovery of bile acid nitrogen from urine	97	98

to that which was employed in the previous experiments with bile acids. A control experiment was carried out with urine to which no hippuric acid had been added. Estimation of the amino nitrogen in the hydrolyzed specimens yielded per 2 cc. (total volume 10 cc.) 0.17 and 0.18 cc. of nitrogen gas, respectively, indicating that hippuric acid does not interfere with the estimation of the bile acids in urine when the latter are estimated according to the above described method.

Several points in connection with the analytical procedure need to be emphasized. On addition of solid magnesium sulfate to

saturation the bile acids separate from their aqueous solution as a semifluid amorphous mass. It is best to retain this in the flask and to wash it several times by decantation with small quantities of a cold saturated solution of magnesium sulfate. Certain precautions must also be taken in the estimation of the amino nitrogen. The presence of the alkali in the hydrolyzed bile influences to a slight extent the nitrogen which is given off by the reagents. In order that this value may be the same for

TABLE IV.

*Recovery of Bile Acids from Urine After the Addition of Human Bile and Ox Bile.*

	Human bile speci- men 1 *	Human bile speci- men 2 *	Human bile speci- men 3 †	Ox bile ‡
Estimation of bile acid nitrogen in the bile.				
Method of Schmidt and Dart.	<i>m</i> <sub>1</sub>	<i>m</i> <sub>2</sub>	<i>m</i> <sub>3</sub>	<i>m</i> <sub>g</sub>
Amino nitrogen after hydrolysis	12.5	9.9	3.7	9.0
“ “ before “	2.5	3.0	1.5	2.1
Nitrogen of bile acids	10.0	6.9	2.2	6.9
Estimation of bile acid nitrogen in urine				
Precipitation by magnesium sulfate				
Amino nitrogen after hydrolysis	11.7	7.6	2.8	9.1
“ “ before “	1.7	0.8	0.7	2.6
Nitrogen of bile acids	10.0	6.8	2.1	6.5
Percentage recovery of bile acid nitrogen from urine	100	98	95	94

\* 5 cc. of human bile per 50 cc. of urine.

† 2 cc. of human bile per 100 cc. of urine

‡ 10 cc. of ox bile per 50 cc. of urine.

the hydrolyzed and unhydrolyzed specimens of bile (thus avoiding the necessity of carrying out the blank estimations) it has been found convenient either to neutralize partially the alkali in the hydrolyzed bile or to add sufficient acetic acid to the reaction vessel to neutralize the alkali which is contained in the specimen of hydrolyzed bile. The method of estimating the bile acids in bile by the method of Foster and Hooper or of Schmidt and Dart is probably not more accurate than 5 per cent. An

allowance for an error of similar magnitude must be made in the method herein described. The method is, however, sufficiently accurate to indicate the magnitude of the value for the output of bile acids in urine.

Opportunity was afforded for the estimation of the bile acid output in the urine from a number of cases of jaundice, the results of which are given in the protocols. These cases cannot be considered as ideal since they do not represent simple cases of bile duct obstruction. Further work will therefore have to be carried out on experimental animals. The results which are given below indicate that in the cases which have been studied only a small quantity of bile acids was excreted through the urine, the maximum output per 24 hours being about 600 mg., calculated as glycocholic acid. The majority of the values fall around 100 mg. Huppert (3), who estimated the output of bile acids in specimens of urine from one case of jaundice, found the output to vary from 28 to 116 mg. Values for bile acid output which are less than 100 mg. per day are undoubtedly subject to considerable analytical error; the values, however, indicate that the amount of bile acids which is excreted is extremely small. Our results likewise indicate that there is no constancy in the amount of bile acids which is eliminated in the urine of a particular individual. Thus, in Case A the output of bile acids was 600 mg. on a certain date and 3 weeks later the output dropped to about 50 mg. The amount of bile acids which was excreted in the urine of Case D was found to be 112 mg. on a certain day and on the following day the output increased to 250 mg. Bischoff (4) believes that man synthesizes about 11 gm. of bile acids per day and Voit (10) on the basis of work which he carried out on a bile fistula dog estimates that the lower limit for the synthesis of bile acids by man is about 11 gm. of dry bile per day. Foster, Hooper, and Whipple (11) found that although the bile acid excretion in a healthy bile fistula dog given a mixed diet will show great variations from day to day, the amount of taurocholic acid which is excreted hour by hour during any given day is fairly uniform. A rough average for the output of taurocholic acid in a bile fistula dog of 30 pounds weight is 100 mg. per hour, or about 2.5 gm. per day. On this basis the amount of bile acids which a man can synthesize lies in the neighborhood of 10 gm.



*Protocols.**Estimations of the Urinary Output of Bile Acids in Cases of Jaundice.*

*Subject A.*—The specimen of urine was obtained from a patient who had carcinoma of the liver. The case was of many months duration and for a period of almost 10 weeks the disease had been progressing rapidly. The patient died about 3 weeks after this specimen of urine had been obtained. The output of bile acid nitrogen per 24 hours, based on estimations which were carried out on a 100 cc. specimen of urine, was 18 mg., which is equivalent to 600 mg. of glycocholic acid.

Another specimen of urine was obtained 10 days later than the above. The output of bile acid nitrogen was 1.6 mg., which represents 53 mg. of glycocholic acid.

*Subject B*—This patient was suffering from carcinoma of the liver and of the gall bladder. The disease had been progressing steadily during the course of 6 weeks during which time the patient had been under observation. He became more jaundiced with time. The total urinary output of bile acid nitrogen was 1 mg. which is equivalent to 33 mg. of bile acid.

*Subject C.*—The urine was obtained from a case of jaundice caused by the obstruction of the common duct due to carcinoma. A week prior to the time at which this specimen was obtained an attempt was made to alleviate this condition by operative procedure, bile pigments were present in the urine in considerable amounts. The urinary output of bile acid nitrogen was found to be 4.6 mg., which represents 152 mg. of glycocholic acid.

*Subject D*—The patient was suffering from an occlusion of the common duct by gall stones. 3 days prior to the time when the specimen of urine was obtained a number of gall stones were passed. Bile pigments were present in the urine in considerable amounts. The total output of bile acid nitrogen in the urine was 3.4 or 113 mg. expressed as bile acid.

Another specimen was obtained 1 day later than the above. The output of bile acid nitrogen was 7.6 mg. which is equivalent to 250 mg. of glycocholic acid.

These values are derived from figures which are based on experimental work which has been carried out on bile fistula animals and represent amounts of bile acids which the animal *can* synthesize rather than those which *are* daily synthesized by normal animals. No data relating to the amount of bile acids which a normal animal synthesizes appear to be available. If we assume that 250 mg. of bile acids represent an average value for the daily urinary excretion in icterus patients we are accounting for only approximately 2 per cent of that which can be synthesized. We realize that this method of calculation is probably extremely

inaccurate; on the other hand, we cannot help but feel that the amounts of bile acids which have been reported in urine do not represent the total production by these individuals. Further evidence for this statement is gained from the work of Huppert (3) who found only traces of bile acids in the urine of a rabbit whose bile duct had been ligated. Further experimental work on this problem is under way.

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# THE DISTRIBUTION OF PENTOSE COMPOUNDS IN THE PANCREATIC TISSUES OF THE LING COD (*OPHIODON ELONGATUS*, GIRARD).

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## INTRODUCTION.

The wide occurrence of a pentose in mammalian glands has long been recognized and the combination in which the sugar occurs has in many cases been identified as guanylic acid (1, 2). The writer has found a similarly wide distribution in the glands of fishes (3) and has shown that, together with guanylic acid, inosinic and adenylic acids are present in the pancreas of the dogfish (4). The recent observation by Jackson (5) of the occurrence of the latter compound in human blood is of particular interest in its bearing on the wide distribution of nucleotides throughout the animal body and their possible physiological significance.

No suggestion as to this physiological significance has been made, but, since, in all cases in which a general survey has been carried out, pentose compounds have been found present in markedly greater quantity in the pancreas than in any other tissue, a functional connection with that organ seems probable.

The pancreas is known to contribute to two distinct metabolic processes, digestion and the maintenance of the carbohydrate equilibrium, and the performance of the latter function has been connected, in the mammalian pancreas, with particular tissues of the organ known as the islets of Langerhans. Determinations of pancreas pentose in fishes have hitherto been confined to certain of the Elasmobranchii (3) and in these the pancreas is a compact organ in which the islets of Langerhans are distributed through the zymogenous tissue in the same way as in that of mammals so that the material taken for analysis contained both

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tissues. Determinations have not previously been made in a fish of any other group largely because the pancreas is in most cases very diffuse and the material, therefore, difficult to collect. In some of the Teleostei certain small glands situated in the mesentery have been shown by Rennie (6) to be homologous with the islets of Langerhans and this has been confirmed by Macleod (7) by the isolation of insulin from them. The zymogenous tissue of the pancreas of these fishes occurs in narrow strips scattered through the mesentery, particularly between the pyloric ceca and adherent to the intestine.

It seemed that some indication of the physiological significance of the pentose compounds of the pancreas might be obtained if it could be found out which of the two essential tissues they particularly characterized by separate determination in each from a suitable teleost fish. The ling cod (*Ophiodon elongatus*, Girard) was used since it was the only such fish available in sufficient quantity to yield enough material for analysis.

### EXPERIMENTAL.

The ling cod is a common food-fish of the coast of British Columbia. As brought in by the fishermen the individual fishes vary in weight from 2 kilos or less to as much as 15 kilos. The largest islet gland is situated in the mesentery near the spleen and is easily recognized. It varies in size with that of the fish, but has never been found larger than a small pea. It is rather conspicuously white, is always encapsulated, and frequently surrounded with a thick coat of fat. Only the principal islets were used and these were taken in most cases from fish of average size. They were carefully excised from freshly killed fish and immediately put into 95 per cent alcohol. On reaching the laboratory they were broken up and pressed under fresh alcohol to insure rapid penetration and the partially ground material was kept under alcohol, which was changed at frequent intervals, for some days. It was then dried, extracted in a Soxhlet with ether, and again dried, first at air temperature and finally at 98°C. The zymogenous pancreatic tissue was cut out of the mesentery in the loop of the intestine to which it adheres rather closely. No islets were to be seen in this tissue, but no histological examination was made,

so that their entire absence is not certain. Samples of this material, and of the islet tissue, were tested in the usual way for the power of inducing the tryptic fermentation of casein. The former rapidly gave a positive reaction for tryptophane, the latter none. The zymogenous tissue was prepared for analysis in precisely the same way as the islets. In the first experiments the dried material was finally ground lightly and put through a fine sieve. This gave a partial separation of the glandular material proper from the investing and connective tissues, but the latter material could not be entirely freed from the former. Pentose was determined separately in both the coarse and the fine material. The yield of fine material from the islet glands was, however, very small; only 0.3 gm. was obtained from the islets extracted from 75 fish. In the second experiments, therefore, for which about the same number of fish was used, the separation was not made.

The method of pentose determination used was Grund's (8) modification of that of Tollens and Krobe and calculation was made to xylose<sup>1</sup> by Grund's formula. The method has been found satisfactory in previous work. It was tested again on pure xylose using quantities to correspond with the amount of pentose anticipated from the small amount of sieved islet tissue available for analysis, and results closely approximating to theory were obtained.

Determinations were also carried out in some other tissues of the ling cod, prepared in the same way as described, for comparison with the pancreatic tissues. In these cases only material passing the fine sieve was taken for analysis.

The results are summarized in Table I.

The islet gland tissues are thus considerably richer in pentose than the corresponding ones from the zymogenous portion of the pancreas. The figures for the latter are, however, high as compared with all the other tissues examined, except the liver. While this may really be characteristic of the zymogenous tissue it is also possible that it is due to the presence of unobserved islets

<sup>1</sup>The actual sugar present in the material is, no doubt, *d*-ribose which has been shown to be the characteristic pentose of the nucleotides by Levene and Jacobs (9), but, since the furfural equivalent of *d*-ribose is uncertain and only comparative results were required, it seemed advisable to calculate on the basis used hitherto.

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therein. The fine cellular material from the islet tissue is considerably the richest in pentose of all those examined. The high pentose content of the liver is in accordance with the results previously obtained for this tissue in fishes (3).

TABLE I

Tissue		Pentose (as xylose).
		<i>per cent</i>
Islet gland (fine material)		2 02
" " (coarse " )		1 63
" " (unsieved)		1 40
Zymogenous pancreatic (fine material)		1 55
" " (coarse " )	...	1 38
" " (unsieved)		1 15
Spleen	..	0 82
Liver		1 62
Ovary		0 86
Kidney		0 65
Heart		0 51
Testis		0 68

### DISCUSSION.

So far, then, as can be judged from the examination of a single species of fish which has the islet and zymogenous tissues separated, it seems justifiable to draw the conclusion that it is to the former, rather than to the latter, than the high pentose content of the pancreas is mainly due in mammals, and in other animals which have the two tissues combined in one organ.

It is hoped to extend the inquiry to other teleost fishes. In the meantime it is interesting to speculate as to how the high pentose content of the islet tissue may be correlated with its function of insulin production.

The action of insulin in controlling carbohydrate metabolism has been ascribed by Winter and Smith (10) to a power of converting ordinary glucose into a highly reactive form<sup>2</sup> in which it readily

<sup>2</sup>Winter and Smith's evidence involves the intervention of a liver enzyme, the action of which is promoted by insulin, to bring about this change. In connection with this observation and the argument which follows, the high pentose content of the liver in all fishes hitherto examined is noteworthy.

undergoes metabolic change. Collip (11) has recently described a hormone, glucokinin, which occurs in plant tissues and has a very similar physiological activity to that of insulin, and which, as he points out, probably functions in the same way, converting glucose into a more reactive form which is necessary for the metabolic processes of the plant. Further, a suggestion has been made by Heilbron and Hollins (12) in connection with plants which is very pertinent to the present inquiry. These authors point out that, while the work of Baly and his associates (13) has shown definitely that only sugars of the  $C_6$  series result directly from solutions of formaldehyde activated by light rays of suitable wave-length and that probably, therefore, only such sugars are formed by photosynthesis in the plant, a very large proportion of the compounds occurring in plant tissues belongs to the  $C_6$  series, and the suggestion is made that these result from the respiratory oxidation of hydroxymethyl-furfuraldehyde which is derived from the primarily synthesized hexose by loss of water. The hypothesis seems plausible and gains force by the discovery of glucokinin, since an activated form of hexose would seem to be necessary to react as suggested.

May not a similar process occur in the animal organism whereby pentose is formed from glucose activated under the influence of insulin? If so, this seems to suggest a connection between the production of the hormone and the localization of pentose compounds in greatest quantity in the tissue in which it is produced.

#### SUMMARY.

1. The tissue of the islet gland of *Ophiodon elongatus* contains considerably more pentose than that of the zymogenous portion of the pancreas.

2. The high pentose content of the pancreas in mammals, Elasmobranchii, etc., is probably, therefore, due to the presence in it of the isles of Langerhans.

3. A connection between the special localization of pentose compounds in the islet tissue and its function of insulin production is suggested.



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# OBSERVATIONS ON THE INORGANIC BASES AND PHOSPHATES IN RELATION TO THE PROTEIN OF BLOOD AND OTHER BODY FLUIDS IN BRIGHT'S DISEASE AND IN HEART FAILURE.

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It has been shown by several writers during the last few years that the inorganic bases of blood serum are remarkably constant in normal individuals,<sup>1</sup> whereas changes have been demonstrated in certain pathological conditions. It has always been anticipated that a decrease in inorganic bases would occur in severe *acidosis* and it has been demonstrated that there actually is a loss of sodium from the blood in this condition in children (Kramer and Tisdall, 2); this element, as the principal inorganic base, is obviously used for neutralizing acid and excreted as the corresponding salt. Changes in the inorganic bases have also been demonstrated in *Bright's disease*. Sodium has been found increased or decreased (Denis and Hobson, 11), potassium and magnesium more or less constant (Myers and Short, 4; Denis and Hobson, 11), and calcium decreased, particularly in the uremic stage (Marriott and Howland, 12; Halverson, Mohler, and Bergeim, 5; Denis and Hobson, 11). The cause of these changes found in Bright's disease is not known; retention is generally accepted as a probable cause of an increased concentration of sodium in serum, while a decreased concentration might be due to water retention. The question of the relation of sodium (and chlorides) to water retention is intimately connected with the problem of edema formation and

<sup>1</sup> *Sodium*: Doisy and Bell (1), Kramer and Tisdall (2). *Potassium*: Kramer and Tisdall (3), Myers and Short (4). *Calcium*: Halverson, Mohler, and Bergeim (5), Meigs, Blatherwick, and Cary (6), Marriott and Howland (7), Kramer and Howland (8), Kramer and Tisdall (9). *Magnesium*: Kramer and Tisdall (9), Denis (10).

is as yet unsettled. Marriott and Howland (12), who discovered the calcium decrease in uremia, pointed out the coincidence of high phosphates and low calcium in this condition. That there might possibly be a relation between the rise in phosphates and the fall in calcium was suggested by the experiments of Binger (13).

As the inorganic bases of the plasma are partly bound to the proteins and the plasma proteins often are low in certain types of kidney disease, it seemed of importance to determine whether there is any parallelism between the changes in the proteins and inorganic bases in Bright's disease. This has been done and we have further, in one case of Bright's disease and in several cases of cardiac decompensation, analyzed different body fluids with various concentrations of protein. Also the relation of the phosphates has been investigated because of the possible connection between phosphate retention and the decrease in calcium.

#### *Methods.*

The inorganic bases were determined in serum by the methods of Kramer and Tisdall (2, 3, 9), the phosphates by the method of Tisdall (14), and the plasma proteins by that of Howe (15). Blood for the determination of the inorganic bases and the phosphates was always taken in the morning before breakfast; no stasis was used, and the blood was collected under paraffin oil, centrifuged at once, and the serum pipetted off. Blood for the determination of plasma proteins was usually collected at the same time unless otherwise stated. Edema fluid was obtained from the subcutaneous tissues of the legs by means of Southey's tubes; narrow rubber tubing filled with sterile oil was attached to the cannula and the fluid was collected in large test-tubes under oil.

#### *Normal Values of the Plasma Proteins, the Inorganic Bases, and the Phosphates of Serum.*

In a previous work from this clinic Linder, Lundsgaard, and Van Slyke (16) found the average normal values of the plasma proteins per 100 cc. of plasma to be as follows: total protein 6.73 gm., albumin 4.12 gm., globulin 2.61 gm., and the albumin:globulin ratio 1.6. The highest value for total protein was 7.45

gm. and the lowest accepted was 6.22 gm., although in one of the normal individuals a value of 5.62 gm. was found with an albumin:globulin ratio of 1.5. A determination made 6 weeks later on the same individual gave 6.84 gm. with the same ratio. The albumin:globulin ratio varied between 1.4 and 2.

Table I shows the values of the inorganic bases and phosphates found in seven normal men. The blood was taken in the morning before breakfast when they were still in bed. The average values of the bases correspond to those of Kramer and Tisdall (2, 3, 9) except that of magnesium, which is a little lower. The values of the phosphates correspond to those found by Tisdall (14).

TABLE I  
*Content of Inorganic Bases and Phosphates in Serum of Normal Adults.*

No	Na		K		Ca		Mg		Inorganic P	
	mg per 100 cc	mm. per liter	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter
1	320	139	19 7	5 0	10 2	2 6	1 7	0 70	3 6	1 2
2	347	150	20 4	5 2	10 3	2 6	1 8	0 74	3 8	1 2
3	335	146	20 1	5 1	9 7	2 4	1 8	0 74	3 4	1 1
4	335	146	19 2	4 9	10 0	2 5	1 8	0 74	3 6	1 2
5	333	145	19 3	4 9	10 4	2 6	1 7	0 70	4 6	1 5
6	331	144	19 1	4 9	10 1	2 5	1 7	0 70	3 7	1 2
7	339	147	21 8	5 6	10 0	2 5	1 9	0 78	4 3	1 4
Average	334	145	19 9	5 1	10 1	2 5	1 8	0 74	3 8	1 2
High	347	150	21 8	5 6	10 4	2 6	1 9	0 78	4 6	1 5
Low.	320	139	19 1	4 9	9 7	2 4	1 7	0 70	3 4	1 1

#### DISCUSSION.

In the classification of Bright's disease we have followed the direction of Volhard and Fahr (17). The material consists of fifteen cases of *Bright's disease* (three cases of nephrosis, ten cases of glomerulonephritis, and two cases of nephrosclerosis) and five cases of *heart failure*. The observations in the cases of Bright's disease with *normal* plasma protein are found in Table II, the results in cases with *low* plasma protein are recorded in Tables III to VII, and those in two cases of *uremia* are found in Table VIII. In patients with heart failure determinations of the proteins, calcium, and phosphates were made upon the blood and

TABLE II  
Inorganic Constituents of Serum of Nephritics with Normal Plasma Proteins.

Case No.	Name. Sex. Age.	Date	Serum										Plasma.			Diagnosis.						
			Na		K		Ca		Mg		Inorganic P		Protein									
			mg per 100 cc	mM per liter	mg per 100 cc	mM per liter	mg per 100 cc	mM per liter	mg per 100 cc	mM per liter	mg per 100 cc	mM per liter	Total	Albu- min	Glob- ulin		A G					
1	A. R. Male. 32	1923 Apr. 24	324	141	21	7	5	6	10	7	2	7	1	7	70	gm per 100 cc	gm per 100 cc		Acute glomerulonephritis.			
2	H. M. Male. 7	" 4														7 82	4 06	3 76	1 1	Chronic glomerulonephritis.		
3	A. F. Male. 34	Oct. 4	340	148	18	3	4	7	10	0	2	5	1	8	0 74	4 6	1 5	7 25	3 66	3 59	1 0	"
4	H. L. Male 57	Mar. 2	330	144	21	2	5	4	10	1	2	5	2	2	0 90	4 7	1 5	7 52	4 17	3 35	1 2	Nephrosclerosis.

TABLE III  
*Serum Inorganic Constituents and Plasma Protein Content.*  
 Case 5. H. J. S. Male. Age 30 years. Diagnosis: Acute nephrosis.

Date.	Serum						Plasma				Remarks.	
	Na		Ca		Mg		Inorganic P.		Protein			
									Total.	Albu- min		Glob- ulin
	mg. per 100 cc	mm per liter	mg. per 100 cc	mm per liter	mg. per 100 cc	mm per 100 cc.						
1943												
Jan. 29	314	137	8.1	2.0	2.5	1.0	4.2	1.4	5.67	2.85	2.82	1 0
Sept. 19	321	140	9.9	2.5	3.2	1.0	3.2	1.0	6.57	3.90	2.67	1 5
												Edema, albuminuria. No symptoms.

Edema, albuminuria.  
 No symptoms.

TABLE IV  
*Serum Inorganic Constituents and Plasma Protein Content.*  
 Case 6 B. S. Male. Age 12 years. Diagnosis: Chronic nephrosis.

Date.	Serum										Plasma				Remarks							
	Na		K		Ca		Mg		Inorganic P		Protein											
											Total.	Albu- min	Glob- ulin	A G								
	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	gm per 100 cc	gm per 100 cc	gm per 100 cc									
1923																						
Mar. 23	326	142	18.9	4.8	7.4	1.9	2.2	0.90	5.0	1.6	4.35	0.99	3.36	0.30								
Apr. 9					7.6	1.9			5.8	1.9	4.34											
" 26					7.9	2.0			5.4	1.7												
May 15					7.9	2.0							1.15	3.28	0.35							
June 15	318	138			7.5	1.9			5.3	1.7	3.77	1.02	2.75	0.37								
Oct. 8					8.1	2.0			5.6	1.8	4.68	1.18	3.50	0.34								

From Mar. 24 to 29, 9 gm. calcium chloride in 30 per cent solution given daily.  
 From Apr 5 to 9, 9 gm. calcium chloride in 30 per cent solution given daily.

TABLE V  
*Inorganic Constituents and Protein Content of Serum (Plasma) and Edema Fluid.*

C. G. D. Male. Age 57 years Diagnosis Nephrosis (amyloid?), tuberculosis of lung.

Date.	Body fluid	Na		K		Ca		Mg		Inorganic P		Protein			Remarks	
		mg per 100 liter cc	mm per 100 liter cc	mg per 100 liter cc	mm per 100 liter cc	mg per 100 liter cc	mm per 100 liter cc	mg per 100 liter cc	mm per 100 liter cc	Total	Albu-min	Glob-ulin	A G			
1923																
Sept. 21	Serum (plasma).	318	138	18.2	4.6	7.9	2.0	1.2	0.50	3.9	1.3	4.48	2.41	2.07	1.16	Tremendous subcutaneous edema.
	Edema fluid.	309	134	12.8	3.3	5.6	1.4	1.8	0.74	3.2	1.0	0.05				
" 28	Serum (plasma).	307	133	19.1	4.9	7.8	2.0	1.5	0.62	4.3	1.4	4.64	0.88	3.76	0.23	Still edematous. Edema fluid drained through cannulas for 5 days.



TABLE VI  
*Serum Inorganic Constituents and Plasma Protein Content in Four Cases of Chronic Glomerulonephritis.*

Case No	Name Sex Age	Date	Serum										Plasma				Remarks													
			Na		K		Ca		Mg		Inorganic P		Protein			A G														
			mg per 100 liter cc	mm per 100 liter cc	mg per 100 liter cc	mm per 100 liter cc	mg per 100 liter cc	mm per 100 liter cc	mg per 100 liter cc	mm per 100 liter cc	Total	Albu- min	Glob- ulin	gm per 100 cc	gm per 100 cc															
8	J O'M. Male. 15	1923 Feb. 3	325	142	25	1	6	4	8	5	2	1	2	7	1	6	7	2	1	4	52	2	85	1	67	1	7	Protein determined Jan. 23.		
9	M. F. Female 25	Jan 27 July 13							6	8	1	7	3	6	1	5	8	7	2	8	5	39	2	13	3	26	0	65	"	Feb. 2.
10	G F Male. 51	June 12 Sept. 12	317	138	20	3	5	2	8	8	2	2					8	0	2	6	5	87	2	92	2	95	1	0	"	
			312	136	17	5	4	5	8	2	2	1	1	50	62		3	2	1	0	4	52	2	19	2	33	0	9	"	
									8	6	2	2					4	6	1	5	4	72	1	48	2	79	0	5	"	
11	J. C. Male 27	Oct. 24	331	144	21	8	5	6	8	5	2	1	2	20	9		5	0	1	6	5	21	2	37	2	84	0	82	"	

*Serum Inorganic Constituents and Plasma Protein Content in Two Cases of Chronic Glomerulonephritis Treated with Calcium Orally.*

Case No	Name Sex. Age	Date	Serum								Plasma				Remarks.		
			Na	K		Ca		Mg		Inorganic P	Protein						
				mg per 100 liter	mm. per 100 liter	mg per 100 liter	mm. per 100 liter	mg per 100 liter	mm. per 100 liter		Total	Albumin	Globulin	A G			
12	J D. Male. 15	1923															
		Jan. 23	315	137			6.9	1.7			6.4	2.0	4.66	2.66	2.0	1.3	Protein determined Jan. 5.
		Feb. 8	337	147	23.1	5.9	8.1	2.0	3.6	1.5	7.0	2.3					Blood taken before calcium administration.
		Mar. 6	332	144	21.4	5.5	8.2	2.1	2.3	0.9	6.3	2.0	4.59	2.27	2.39	1.0	From Feb. 8 to Mar. 12, 20 gm. calcium lactate in 5 per cent solution daily.
13	S. L. Male. 14	Oct 26	320	139	22.0	5.6	7.8	2.0	2.4	1.0	6.7	2.2	4.37	2.03	2.34	0.87	
		Feb. 1	311	135	22.7	5.8	7.7	1.9	2.2	0.9	8.0	2.6	4.52	2.77	1.75	1.6	Protein determined Jan. 26.
		" 14	316	137			8.0	2.0	2.1	0.9	7.9	2.6					Calcium lactate treatment started Feb. 6.
		" 26	347	150	22.5	5.8	8.8	2.2			6.3	2.0					20 gm. calcium lactate in 5 per cent solution daily since Feb. 6
		" 26															From Feb. 20 to 26, 12 gm. calcium chloride in 30 per cent solution daily.
		Mar. 27	331	144	23.2	5.9	8.1	2.0	1.7	0.7	7.3	2.4	5.15	2.54	2.61	1.0	Calcium lactate daily as before since Feb. 26.
		June 13	321	140	18.7	4.8	6.3	1.6	1.9	0.8	9.0	2.9	4.87	2.33	2.54	0.9	No calcium treatment since Mar. 28.
		" 19						6.3	1.6		8.6	2.8					

[illegible]

TABLE IX.  
*Inorganic Constituents and Protein Content of Different Body Fluids.*  
 Case 16. M. D. Male. Age 46 years Diagnosis Syphilis, aortic insufficiency.

Date.	Body fluid	Na		K		Ca		Mg		Inorganic P		Protein				Remarks	
		mg per 100 liter	mm per 100 cc	mg per 100 liter	mv per liter	mg per 100 liter	mm per liter	mg per 100 cc	mm per 100 liter	mg per 100 cc	mm per 100 liter	Total	Albumin	Globulin	A G		
1923 Mar. 29	Serum	360	156	22	0 5	6 10	4 2	6 1	7 0	70	3 6	1 2	6 12	2 78	3 34	0 83	Protein determined in plasma.
Apr. 6	Ascitic fluid																Protein determined in plasma.
	Edema	351	153	15	9 4	0 6	3 1	6 1	1 0	45	3 6	1 2	0 29				
	Serum	334	145	21	8 5	6 9	0 2	3 1			3 4	1 1	5 72	2 53	3 19	.0 80	
May 14	Chest fluid.																Protein determined in plasma.
	Serum	330	143	21	2 5	4 10	0 2	5 1	5 0	62	3 9	1 3	7 87	0 87	0 85	1 0	
														4 01	3 86	1 1	Protein determined in plasma.

TABLE X  
*Inorganic Constituents and Protein Content of Serum (Plasma) and Edema Fluid.*  
 Case 17. E. G. Male Age 41 years Diagnosis: Syphilis, valvular disease

Date	Body fluid	Na		K		Ca		Mg		Inorganic P		Protein				Remarks										
		mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	Total	Albumin	Globulin	A G											
1923																										
May 9	Serum	305	132	23	6	0	8	5	2	1	40	57	3	7	1	2	5	82	2	48	3	34	0	7	Protein determined in plasma.	
June 8	Edema fluid.	305	132	18	8	4	8	5	8	1	5		0	24												Protein determined in plasma.
	Serum.	314	137	20	0	5	1	8	5	2	1	40	57	3	9	1	3	5	94	2	57	3	37	0	7	

TABLE XI  
*Calcium, Phosphates, and Protein Content of Different Body Fluids in Three Cases of Heart Failure.*

Case No	Name Sex Age	Date	Body fluid	Ca		Inorganic P	Protein				Remarks
				<i>mg per 100 cc</i>	<i>mm per 100 liter</i>		Total	Albumin	Globulin	A G	
18	F K. Male 51	1923 Apr 10	Serum (plasma). Ascitic fluid	<i>mg per 100 cc</i>	<i>mm per 100 liter</i>	<i>mg per 100 cc</i>	<i>gm per 100 cc</i>	<i>gm per 100 cc</i>	<i>gm per 100 cc</i>		Nephrosclerosis, myocardial insufficiency.
				9.3	2.3	3.0	1.0	6.24	2.94	3.30	0.89
		" 17 Oct 5	Edema Serum (plasma) " ( " )	6.9	1.7	3.1	1.0	2.24	1.04	1.20	0.83
				5.1	1.3	-	-	0.35	-	-	-
19	H. R. Male. 67	May 19	" ( " ) Chest fluid.	8.4	2.1	4.1	1.3	5.17	2.61	2.56	1.0
				9.7	2.4	3.6	1.2	6.87	3.64	3.23	1.1
				9.8	2.5	4.4	1.4	6.57	3.35	3.22	1.0
20	L. P. Female. 10	June 9	" ( " ) Chest fluid	6.8	1.7	4.0	1.3	1.76	1.02	0.74	1.4
				8.4	2.1	2.9	0.9	5.95	3.25	2.70	1.2
				5.9	1.5	3.1	1.0	1.88	1.02	0.86	1.2

Valvular disease

fluid obtained at the same time from the pleura or peritoneum; edema fluid also was obtained in three cases and similarly analyzed. In two of the cases the other inorganic bases were determined. The results in these two latter cases may be seen from Tables IX and X, while the observations in the other three cases are recorded in Table XI. The relationship between calcium and protein content of different body fluids is seen from Fig. 1.

It seems obvious, from all the observations in Bright's disease, that there is a parallelism between the changes in plasma protein and in calcium content of serum in Bright's disease. In every

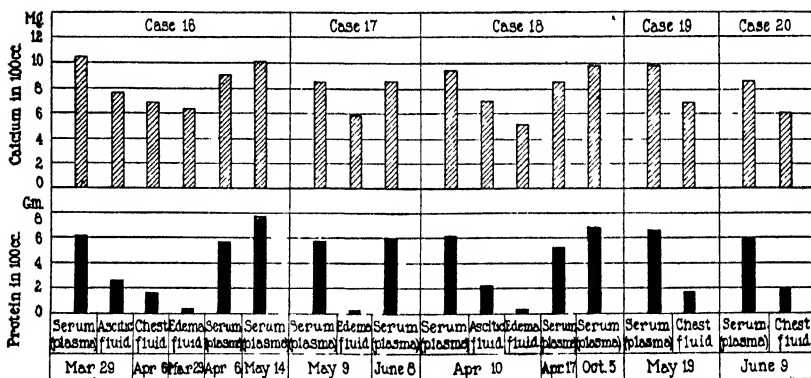


FIG 1. Relation between calcium and protein per 100 cc. of various body fluids in patients with cardiac hydrops

case in which low protein was found, there was also a decrease in the calcium content. A low plasma protein concentration was observed in some of the heart cases, and here, too, the calcium content of the serum was diminished. Subsequent determinations showed that when the protein concentration returned to normal, the calcium content rose to normal also. This is seen from Fig. 2 (nephrosis case) and from Fig. 1, Cases 16 and 18 (heart cases). Likewise, whenever a lowered serum calcium was demonstrated the plasma protein was found to be decreased. In two cases of uremia, however, the calcium decrease seemed out of proportion to the drop in protein when compared with the other cases in which renal function was relatively unimpaired. This is seen especially well in Case 14 (Table VIII). However, in these

cases of uremia there was an enormous retention of phosphates and in Case 14 (Table VIII) the decrease in calcium was proportional to the increase in phosphate. In repeating Binger's experiments (13) upon the effects of intravenous injections of phosphates on the serum calcium, Tisdall (18) found that when the decrease of calcium was established the phosphates increased to about the same degree as observed in our uremia cases. It seems very likely that the phosphate retention is responsible for the excessive drop of serum calcium in uremia.

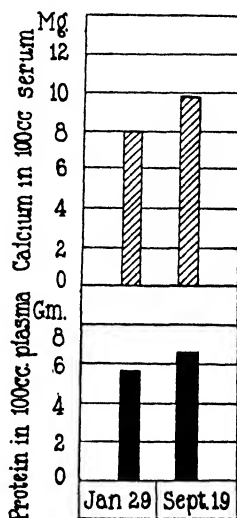


FIG. 2.

FIG. 2. Relation between calcium and protein in 100 cc. of serum (plasma) in a case of acute nephrosis at the time of admission and when recovered (Case 5).

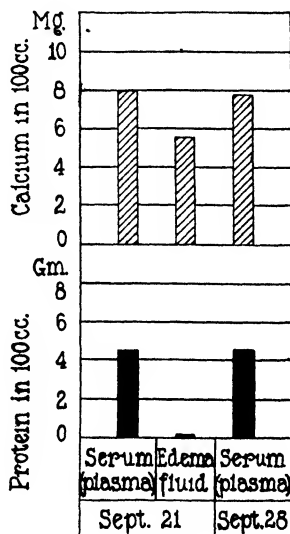


FIG. 3.

FIG. 3. Relation between calcium and protein per 100 cc of serum and edema fluid in Case 7.

The observations on the calcium content of blood and different extravascular body fluids obtained from one case of nephrosis (Fig. 3) and from five cases of heart failure with anasarca show a close parallelism between the calcium content and the protein content. It seems justifiable to conclude that the cause of the drop in serum calcium observed in Bright's disease without uremia is the decrease in the plasma protein. The other inorganic



show an irregular behavior and may vary in either direction.<sup>2</sup> The deproteinization of the blood does not seem to affect these bases in any characteristic way.

It is still an open question in what form calcium exists in the blood serum. From dialysis and ultrafiltration experiments *in vitro* it seems to be certain that 50 to 70 per cent of the blood calcium is diffusible (Rona and Takahashi, 19; Cushny, 20; von Meysenbug, Pappenheimer, Zucker, and Murray, 21; Neuhausen and Pincus, 22). How much of this diffusible calcium is ionized is unsettled. The experiments of Neuhausen and Marshall (23),

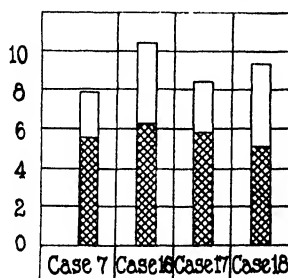


FIG. 4 Relation between diffusible (lower part) and non-diffusible (upper part) serum calcium in Cases 7 (nephrosis), 16, 17, and 18 (heart failure).

who worked with calcium amalgam electrodes, indicate that only 15 to 25 per cent is ionized. In one of our cases of nephrosis (Case 7) and in three of the cases of heart failure, in which the calcium was determined in blood and in the practically protein-free edema fluid, the dialysis "experiment" is performed *in vivo* by nature, and it shows that 55 to 70 per cent of the serum calcium is diffusible and is found in the edema fluid (in Case 7, Fig. 3, 70 per cent; in Cases 16, 17, and 18, Fig. 1, 60, 68, and 55 per cent, respectively). The diffusible quota of the total serum calcium in these cases is seen from Fig. 4, which is charted from the data of Figs. 1 and 3. Whether the other 30 to 45 per cent are

<sup>2</sup> The magnesium method is not accurate enough to detect smaller variations. The potassium values are given with reservation as we often have encountered difficulties with the method. Sometimes a precipitate, which is not potassium cobalti-nitrite occurs, especially in ascitic or chest fluids.

bound to protein or to other organic material is not known; but the fact that blood calcium is reduced in cases of Bright's disease in which a low plasma protein concentration is found, and the parallelism between the calcium and the protein content of different body fluids indicate that the non-diffusible calcium of the blood probably is bound to protein. This protein-bound calcium is apparently not ionized to the same degree as the diffusible calcium; if the results of Neuhausen and Marshall (23) are correct, most of the protein-bound calcium must be unionized. In the following paper we have discussed the biological significance of the protein-bound calcium. A loss of blood calcium through loss of blood protein will probably have no immediate consequence in regard to lack of specific calcium action; as the amount of ionized calcium remains unchanged, tetany will probably not occur from that cause.

It was observed also that potassium and magnesium were markedly lower in the edema fluid than in the blood serum. We do not feel justified in drawing any conclusion from these few observations for reasons stated above.<sup>2</sup> It will be noted that administration of large doses of calcium by mouth had very little effect in raising the calcium content of the serum (Cases 6, 12, and 13).

#### SUMMARY.

In fifteen cases of Bright's disease the inorganic bases, the phosphates, and the plasma proteins have been studied. In four non-uremic cases in which the plasma protein was normal, the inorganic bases were also normal. In nine cases in which the plasma protein was diminished a marked drop in the calcium content was found, while sodium was normal or slightly decreased, and potassium and magnesium varied in both directions. In two cases of uremia in which the plasma protein was but slightly decreased, the drop in calcium was more marked than in the non-uremic cases; a great retention of phosphates was found in these two cases.

In five cases of heart failure with dropsy the calcium content of different body fluids (serum, chest fluid, ascitic fluid, edema fluid) decreased proportionately to the protein content.

## CONCLUSIONS.

The decrease in serum calcium found in non-uremic cases of Bright's disease without phosphate retention parallels the decrease in plasma protein. It appears that phosphate retention may cause a drop in the serum calcium in Bright's disease. The diffusible serum calcium is from 55 to 70 per cent of the total calcium. The non-diffusible portion is probably bound to the plasma proteins.

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## THE RELATION BETWEEN CALCIUM AND PROTEIN OF SERUM IN TETANY DUE TO PARATHYROIDECTOMY.

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It has been shown in the preceding paper (1) that 30 to 45 per cent of the serum calcium is non-diffusible and bound to the plasma protein, and further that in Bright's disease the low plasma protein concentration causes a decrease in the serum calcium. In parathyroid tetany there is a marked decrease in the blood calcium and it has been shown in recently published experiments (2) that all the symptoms of parathyroidectomized dogs are due to calcium deficiency, as first maintained by MacCallum and Voegtlin (3). Several authors have tried to determine whether it is the non-diffusible or the diffusible part of the blood calcium which is diminished in parathyroid insufficiency. Von Meysenbug and McCann (4), working with a compensation dialysis method, found the diffusible calcium of the serum in dogs with parathyroid tetany to be between 60 and 70 per cent of the total calcium present in serum, which corresponds to the figure found by von Meysenbug, Pappenheimer, Zucker, and Murray (5) for normal blood. They conclude, therefore, that the reduced serum calcium in experimental tetany is not due to a lowering of the diffusible as contrasted with the non-diffusible form. Cruickshank (6) came to the conclusion that the colloidal calcium is greatly reduced in experimental tetany, since he found the diffusible calcium to amount to 94 per cent of the total calcium in severe parathyroid tetany. He ascribes the loss of colloidal calcium to a rapid protein disintegration. The cause of the loss of calcium in parathyroid insufficiency is not known.

### EXPERIMENTAL.

In order to find out whether the drop in blood calcium found in parathyroid tetany is due to a lowering of the plasma protein,

as in Bright's disease, we have followed the serum calcium and the plasma protein from day to day in two dogs in which the parathyroids (and part of the thyroids) were removed. Phosphates and the other inorganic bases were also determined. The methods were the same as those used in the preceding paper (1). The parathyroids were removed under morphine (0.04 gm.) and ether anesthesia. From Tables I and II it will be seen that the calcium decreases following the operation, and that when it has passed 7 mg. per 100 cc. of serum (1.75 mm.) tetany occurs. As the calcium falls still lower the tetanic symptoms become more severe. *The plasma proteins remain constant.* The other inorganic bases show no changes,<sup>1</sup> but the phosphates increase.

TABLE I

*Inorganic Constituents of Serum and Plasma Protein Following Parathyroidectomy*

Dog 1 Weight 17 kilos

Date	Serum								Plasma protein			Remarks
	Na		Ca		Mg		Inorganic P		Total	Albumin	Globulin	
	mg per 100 cc.	mm per liter	mg per 100 cc.	mm per liter	mg per 100 cc.	mm per liter	mg per 100 cc.	mm per liter	gm per 100 cc.	gm per 100 cc.	gm per 100 cc.	
1923 June 26	328	143	11.2	2.8	1.4	0.58	3.05	1.0	5.72	3.14	2.58	June 27, removal of four parathyroids.
" 28 1.30 p m.	330	143	6.9	1.7	1.4	0.58	4.7	1.5	5.80	2.95	2.85	Tetany; twitchings of shoulders and fore legs
June 29	336	146	6.2	1.5	1.3	0.53	5.1	1.6	5.50	2.90	2.60	More pronounced tetany. Refuses food.

<sup>1</sup> The results of the potassium determinations were not reliable as a precipitate occurred which did not dissolve by boiling with the permanganate solution and sulfuric acid.

TABLE II  
*Inorganic Constituents of Serum and Plasma Protein Following  
 Parathyroidectomy.*

Dog 2. Weight 12 kilos.

Date	Serum								Plasma protein			Remarks
	Na		Ca		Mg		Inorganic P		Total	Albumin	Globulin	
	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	mg per 100 cc	mm per liter	gm per 100 cc	gm per 100 cc	gm per 100 cc	
1923 July 3	354	154	10 1	2 5	1 5	0 6	1 3	4 1	5 90	2 80	3 10	Parathyroid- ectomy performed after blood was taken for analysis.
" 4	360	157	9 05	2 2	1 4	0 58	4 3	1 4	5 95	2 79	3 16	No tetany.
" 5			7 5	1 9			3 3	1 1	5 60	2 50	3 10	" "
" 6	327	142	7 6	1 9	1 5	0 6	1 5	4 1	7 5	2 85	2 85	" "
" 10			4 9	1 2	1 1	0 45	5 0	1 6	6 00	2 90	3 10	Tetany ob- served the evening of July 9 July 10, violent tetany.

#### DISCUSSION.

It is apparent from these results that the decrease in blood calcium which occurs after removal of the parathyroids is not due to a lowering of the plasma protein. This result is what might be expected from theoretical considerations. In parathyroidectomized dogs the calcium values of the serum go down to 7 mg. per 100 cc. (1.75 mm.) before tetany occurs and values between 3 and 4 mg. (0.8 to 1 mm.) are frequently found (2). If the protein-bound calcium constitutes at most from 30 to 45 per cent of the total calcium, the theoretically maximum decrease of serum calcium, due to disappearance of plasma protein, never can exceed 4.5 mg. of calcium per 100 cc. (assuming 10 mg. as the average normal). As the plasma protein remains constant during development of tetany there is no reason to believe that in tetany there

is a primary loss of non-diffusible calcium. But as a result of a lowering of the concentration of diffusible (and ionized) calcium, which probably takes place in parathyroid tetany, the protein-bound calcium will also be lowered, as the equilibrium will be disturbed. The results of von Meysenbug and McCann (4) and those of Cruickshank (6) are easily explained in this way. When the lowering of the serum calcium has passed a certain point, probably all the remaining calcium can be accounted for in dialysis experiments with the reservations caused by the Donnan law of membrane equilibrium. Thus the protein-bound calcium acts as a reserve which will furnish a new supply of ionized calcium, if this important part of the blood calcium is diminished. The symptoms of calcium deficiency, such as tetany and increased irritability of muscle and nerve tissues, are due to deficiency of calcium ions (Loeb, 7); in Bright's disease (without uremia) the decrease in blood calcium, being caused by the diminished concentration of plasma protein, causes no diminution of calcium ions and, therefore, produces no calcium deficiency symptoms. The loss of blood calcium in tetany, on the other hand, is primarily a loss of calcium ions and will, therefore, produce symptoms when the decrease in blood calcium has passed a certain point. The importance of this discrimination between non-diffusible, protein-bound calcium and the diffusible and ionized calcium must be emphasized. In all conditions in which a calcium decrease in the blood has been demonstrated, the plasma protein ought to be determined as an indication of which of the two calcium fractions is primarily decreased.

#### SUMMARY.

In two dogs in which the parathyroids were removed and which developed tetany, the plasma protein remained constant, while the serum calcium decreased. Tetany occurred when it had passed below 7 mg. per 100 cc. of serum. This is taken as an indication that the calcium decrease in tetany is not due to a primary decrease in the protein-bound calcium, but is caused by a decrease in the diffusible (and ionized) fraction. It appears probable from this and the preceding paper that a parallel fall in both protein and Ca in the plasma (such as was observed in nephritis), with pH and other significant factors normal, affects chiefly the

non-ionized Ca; while a fall in Ca with continued normal protein content affects chiefly the ionized Ca and may lead to tetany.

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## A COLOR TEST FOR CHLOROFORM AND CHLORAL HYDRATE.

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(Received for publication, October 20, 1923)

Solutions containing chloroform, bromoform, iodoform, or chloral hydrate when added to a mixture of aqueous sodium or potassium hydroxide and pyridine produce on heating a deep red coloration.

The formation of this color seems to be dependent on the presence of the  $R-CHHg$  ( $Hg=Cl, I, \text{ or } Br$ ) group, as acid chlorides, inorganic chlorides, monochlorobenzene, and benzal chloride do not produce it. Chlorinated toluene (containing benzotrichloride) reacts similarly to chloroform, etc., while the reaction product of acetone and  $PCl_5$  does not produce the color.

The colored compound is soluble in pyridine. It loses its color in alcohol. Acids destroy the color which may not be reproduced by rendering the solution alkaline again.

This test might possibly be used advantageously in conjunction with any method of qualitative analysis based on the formation of  $CHCl_3$ ,  $CHBr_3$ , or  $CHI_3$ . In the detection of citric acid by the method of Stahre,<sup>1</sup> or Wöhlk<sup>2</sup> in which bromoform is produced, this test shows presence of the bromo form evolved by small amounts of citric acid or its salts.

Quinoline and piperidine do not produce the same color with trihalides and alkalis.

### *Method.*

3 to 5 cc. of concentrated (17 to 25 per cent) NaOH solution in a test-tube are superimposed with a layer 2 mm. thick of pyri-

<sup>1</sup> Stahre, L., *J. Soc. Chem. Ind.*, 1896, xv, 53.

<sup>2</sup> Wöhlk, A., *Z. anal. Chem.*, 1902, xli, 77.

dine. A small quantity of the substance or a drop of the solution to be tested is added and the contents of the tube are raised to the boiling point, shaking well to avoid bumping. If the color has not appeared when the mixture has boiled a few seconds, the test-tube should be shaken vigorously and then held still until the pyridine layer has risen to the surface. If  $\text{CHCl}_3$ , etc., are present the pyridine will be colored from a pink to a clear deep red.

0.1 cc. of 0.0014 per cent solution of chloral hydrate gave dark red—positive for quantities below 0.014 mg.

$\text{CHCl}_3$  was detected in amounts of 0.005 mg.

#### SUMMARY.

The red color produced on heating chloroform, bromoform, iodoform, and chloral hydrate with aqueous  $\text{NaOH}$  and pyridine may be used to identify these compounds; and, further, to identify substances which produce these compounds under given conditions and in amounts inferior to 0.005 mg.

Piperidine and quinoline do not produce this color.

The test may apply only to substances which contain the grouping  $\text{R-CHlg}_3$ .

## THE DIRECT DETERMINATION OF THE SECONDARY PHOSPHATE.\*

BY I. NEWTON KUGELMASS AND CARMEN ROTHWELL.

(From the Department of Pediatrics, Yale University, New Haven)

(Received for publication, October 18, 1923.)

The present method for the direct determination of secondary phosphate was necessary to afford sufficient experimental data for the evaluation of the constants in systems containing the ions,  $\text{Ca}^{++}$ ,  $\text{H}^+$ ,  $\text{HCO}_3^-$ ,  $\text{CO}_3^{--}$ ,  $\text{HPO}_4^-$ ,  $\text{H}_2\text{PO}_4^-$ , and  $\text{PO}_4^{==}$  (1). Such a method is suitable to determine the distribution of the phosphates in solutions in which the hydrion concentration cannot be determined either electrometrically or colorimetrically. A reagent suitable for the precipitation of secondary phosphate requires that: (a) it precipitate the secondary phosphate ion completely and quantitatively at any hydrion concentration of the solution; (b) it shift no equilibria in the direction of conversion of the secondary phosphate ion from the reserve of primary phosphate; and (c) it precipitate no primary phosphate ion.

*Reactions of Calcium Salts with Phosphates.*—The calcium salts that precipitate the phosphate ions are: (1) those of the weak acids which precipitate the primary phosphate but not the secondary, *e.g.*  $2\text{NaH}_2\text{PO}_4 + 3\text{CaCO}_3 \rightleftharpoons \text{Ca}_3(\text{PO}_4)_2 + \text{Na}_2\text{CO}_3 + \text{H}_2\text{O} + 2\text{CO}_2$ ; (2) those of the weak volatile acids which do not precipitate the secondary phosphate; they precipitate the primary phosphate and also displace the equilibrium of the system in the direction of formation of secondary phosphate ion from the primary due to the escape of a volatile product during the reaction, *e.g.*  $2\text{NaH}_2\text{PO}_4 + \text{CaS} \rightleftharpoons \text{Na}_2\text{HPO}_4 + \text{CaHPO}_4 + \text{H}_2\text{S}$ ; (3) those of strong acids which react with secondary phosphate, without affecting the primary phosphate, *e.g.*  $4\text{Na}_2\text{HPO}_4 + 3\text{CaSO}_4 \rightleftharpoons \text{Ca}_3(\text{PO}_4)_2 + 2\text{NaH}_2\text{PO}_4 + 3\text{Na}_2\text{SO}_4$ . The phosphorus of the secondary phosphate is equally distributed between the tricalcium

\* Aided by a grant from the Loomis Fund.

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phosphate and the monosodium phosphate. The primary phosphate is formed as a result of this reaction and the presence of  $\text{H}_2\text{PO}_4^-$  in the system does not shift the equilibrium to form  $\text{HPO}_4^-$  because the reverse process occurs. Therefore, precipitation of secondary phosphate by  $\text{CaSO}_4$  satisfies the necessary conditions given above. Chemical and physicochemical studies were therefore made of the exact reacting components in such an equilibrium and its application to the quantitative determination of secondary phosphate in aqueous solutions.

*Preparation of Materials.*—Conductivity water was obtained by redistilling water through a block tin condenser from alkaline permanganate.

Calcium sulfate was prepared by addition of dilute sulfuric acid to calcium chloride. The addition was gradual to prevent the formation of coarse lumps of sulfate. The precipitate was washed with water on a Buchner funnel and dried by suction. The hydron concentration of the saturated solution of  $\text{CaSO}_4$  is  $0.26 \times 10^{-7}$ , due to slight hydrolysis. The ideal reagent should be neutral in reaction, but its slight alkalinity causes no difficulty because  $\text{CaSO}_4$ , which has no buffer value, is added to phosphates which buffer well.

Monopotassium phosphate ( $\text{KH}_2\text{PO}_4$ ) — the c p salt was recrystallized twice from distilled water and dried to constant weight at  $120^\circ$ .

Disodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ) was prepared by the method of Hansen (2)

*Analysis of the Precipitate from  $\text{CaSO}_4$  and  $\text{Na}_2\text{HPO}_4$ .*—A 0.03 N  $\text{CaSO}_4$  is a saturated solution at  $25^\circ$  and contains 2.04 gm. per liter. 100 cc. of this solution were added to 100 cc. of a 0.03 N  $\text{Na}_2\text{HPO}_4$ , warmed on a hot-plate at  $60^\circ$ , and filtered hot. The resultant precipitate was washed with half saturated  $\text{CaSO}_4$  at  $60^\circ$ , then with warm distilled water, and finally dried to constant weight. The heating was not carried beyond  $60^\circ\text{C}$ . because gypsum is the stablest of the calcium sulfates and in aqueous solution remains stable to  $66^\circ$  when it passes into the anhydride. The averages of two determinations yielded 54.0 per cent CaO and 45.1 per cent  $\text{P}_2\text{O}_5$  as the percentage composition of the precipitate. These values are in good agreement with those theoretically required for  $\text{Ca}_3(\text{PO}_4)_2$ , 54.2 per cent CaO and

45.8 per cent  $P_2O_5$ . The average weight of precipitate was 0.0716 gm. or 93 per cent of the theoretical value.

*Conductivity Measurement of the Reacting System:  $CaSO_4 + Na_2HPO_4$ .*—To determine what reaction takes place between the two components and their ions, conductivity measurements were made (3). The usual Kohlraush method was used for systems containing varying quantities of saturated calcium sulfate and 0.01 N  $Na_2HPO_4$  immediately after mixing in the thermostat at 25°, and in systems after bringing to 60° on a hot-plate and cooling to 25° in the thermostat. The cell of the dipping electrode type, standardized by a 0.01 N KCl, showed a cell constant of 0.225, and was kept in the thermostat during the determination.

TABLE I  
*Specific Conductivity of Mixtures of  $CaSO_4$  and  $Na_2HPO_4$  at 25°.*

Saturated $CaSO_4$ solution		Specific conductance in mhos ×	
		Immediately after mixing	After warm. 60°C
cc	mg		
5	10	3 34	3 3
10	20	3 48	3 4
15	30	3 70	3 6
20	40	3 88	3 7
25	50	4 02	3 9
30	60	4 38	4 2
35	70	4 72	4 6
40	80	4 93	4 8

The data in Table I show that equilibrium is rapidly attained with  $CaSO_4$  regardless whether or not the transformation of the secondary phosphate is complete. When the data are plotted with conductivities as ordinates and varying volumes of  $CaSO_4$  as abscissæ the curve shows a distinct break at 50 mg. of  $CaSO_4$  and 70 mg. of  $Na_2HPO_4$ . Calculation from the equation requires 51 mg. of  $CaSO_4$  for the ratio of  $4Na_2HPO_4:3CaSO_4$ . Hence this equation, from among others possible, is established. This equilibrium has been previously shown by analysis to yield 93 per cent precipitation.

*The Conditions for Complete Precipitation of Secondary Phosphate.*—A study was made to determine the optimum conditions

for quantitative transformation of the secondary phosphate. An excess of  $\text{CaSO}_4$  shifts the equilibrium to complete precipitation according to the mass action principle and prevents the formation of the unstable system:  $4\text{CaSO}_4 + 4\text{K}_2\text{HPO}_4 \rightleftharpoons \text{Ca}_3(\text{PO}_4)_2 + \text{Ca}(\text{H}_2\text{PO}_4)_2 + 4\text{K}_2\text{SO}_4$  and also prevents the hydrolysis of the normal  $\text{Ca}_3(\text{PO}_4)_2$ .

The optimum excess of  $\text{CaSO}_4$  necessary for complete precipitation of M/15  $\text{Na}_2\text{HPO}_4$  was determined. To 5 cc. of  $\text{Na}_2\text{HPO}_4$ , containing 10 mg. of P, increasing quantities of saturated  $\text{CaSO}_4$  were added. The solution was shaken and warmed on a hot-plate at  $60^\circ\text{C}$ . for a few minutes. The precipitate was filtered hot, washed with warm half saturated  $\text{CaSO}_4$  solution, and finally dissolved on the filter paper with warm 10 per cent  $\text{HNO}_3$ . The

TABLE II

*The Effect of Increasing Concentrations of  $\text{CaSO}_4$  on the  $\text{HPO}_4$  Determination.*

$\text{CaSO}_4$	$\frac{1}{2} \text{HPO}_4$	P in precipitate	P in filtrate	Error in precipitate
cc	mg P	mg	mg	per cent
5	5	4.2	6.0	16.0
10	5	4.4	6.1	12.0
15	5	4.7	5.4	6.0
20	5	4.8	5.0	4.0
25	5	5.1	5.0	2.0
30	5	5.0	5.0	0.0
35	5	5.0	5.0	0.0

volume of the resulting solution was made up to 25 cc. Duplicate phosphate determinations were then made colorimetrically on both filtrate and dissolved precipitate by Briggs' modification of the Bell-Doisy method (4).

The data, given in Table II, show that an amount of  $\text{CaSO}_4$  equivalent to five times the secondary phosphate gives the theoretical values for complete precipitation of the phosphate.

In order to determine whether this procedure was valid in the presence of primary phosphate a similar series of determinations was carried out following the same procedure with the varying mixtures of primary and secondary phosphate as indicated in Table III.

The data show that in the ratio of 1:19 or 19:1 primary phosphate does not interfere with the determination of secondary

phosphate. The minimum amount of  $\text{HPO}_4$  that can be recovered quantitatively is about 0.05 mg. with an error of  $\pm 5$  per cent.

*Procedure for the Determination of Secondary Phosphate.*—To 1 cc. of a solution containing at least 0.05 mg. of P as  $\text{HPO}_4$  in a centrifuge tube add 5 cc. of saturated  $\text{CaSO}_4$ . Put the tube in a water bath at  $60^\circ$  for a few minutes. Centrifuge, blow off the supernatant liquid, and wash the precipitate twice with warm half saturated  $\text{CaSO}_4$  solution. Dissolve the final residue with 10 per cent  $\text{HNO}_3$ . Make up the volume to 10 cc. and in an aliquot portion determine the phosphorus by Briggs' modification of the Bell-Doisy method (4). The value of phosphorus obtained colorimetrically multiplied by two equals the phosphorus of the secondary phosphate.

TABLE III

*The Effect of Increasing Concentrations of  $\text{H}_2\text{PO}_4$  on the  $\text{HPO}_4$  Determination.*

10 cc of solution of phosphates				CaSO <sub>4</sub>	P	Calculated P	Error
(HPO <sub>4</sub> )		(H <sub>2</sub> PO <sub>4</sub> )					
cc	mg	cc	mg				
0 25	0 5	9 75	19 50	50	0 24	0 25	5 0
2 00	4 0	8 00	16 00	50	2 10	2 00	5 0
5 00	10 0	5 00	10 00	50	5 12	5 00	2 3
8 00	16 0	2 00	4 00	50	8 05	8 00	0 6
9 50	19 0	0 50	1 00	50	9 65	9 50	1 6

The method has likewise given reproducible results upon addition of 5 cc. of aqueous saturated  $\text{CaSO}_4$  to 1 cc. of solution containing at least 0.05 mg. of P as  $\text{HPO}_4$  in a small Erlenmeyer flask, warming at  $60^\circ$ , filtering on hard filter paper, and washing several times with warm half saturated  $\text{CaSO}_4$ . The precipitate is finally dissolved with 10 per cent  $\text{HNO}_3$  and collected in a measuring flask, and an aliquot portion used for the phosphorus determination.

If a simultaneous determination of total phosphate is made, the ratio of the primary and secondary phosphates and hence the hydron concentration can be calculated.

## SUMMARY.

Saturated  $\text{CaSO}_4$  is a precipitant of the secondary phosphate forming calcium orthophosphate according to the equilibrium



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equation:  $4\text{K}_2\text{HPO}_4 + 3\text{CaSO}_4 \rightleftharpoons \text{Ca}_3(\text{PO}_4)_2 + 2\text{KH}_2\text{PO}_4 + 3\text{K}_2\text{SO}_4$ . This equation was confirmed by analysis of the precipitate as well as by conductivity measurements. A simple method has been devised for the direct determination of the secondary phosphate containing at least 0.05 mg. of P as  $\text{HPO}_4$  in the presence of twenty times the amount of primary phosphate in solution, with an error of  $\pm 5$  per cent.

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# THE DETERMINATION OF THE EQUILIBRIA INVOLVING CALCIUM, HYDROGEN, CARBONATE, BICARBONATE, AND PRIMARY, SECONDARY, AND TER- TIARY PHOSPHATE IONS.\*

BY I. NEWTON KUGELMASS AND A. T. SHOHL

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(Received for publication, October 18, 1923)

Quantitative equilibrium relations have been developed for the ions,  $\text{Ca}^{++}$ ,  $\text{H}^+$ ,  $\text{HCO}_3^-$ ,  $\text{CO}_3^{--}$ ,  $\text{HPO}_4^-$ ,  $\text{H}_2\text{PO}_4^-$ , and  $\text{PO}_4^{--}$ , and expressed in a single equation. The equilibrium constants have been determined experimentally under conditions within physiological limits. This system of calcium salts has been selected because (a) their ions are involved in the physiology and pathology of bone calcification and of nervous irritability (*e.g.* rickets and tetany); (b) these ions have not been included in other studies of electrolyte equilibria (1) since the calcium and phosphorus are too small to affect the osmotic and hydron equilibria of the blood. However, the hydron concentration directly affects the ionization of the calcium salts of this system. The general character of this equilibrium is such that a change made in the concentration of one of the components produces measurable changes in all the others. With a knowledge of the equilibrium constants of these ions one can predict changes in the ionic concentrations in the blood as well as in other aqueous systems.

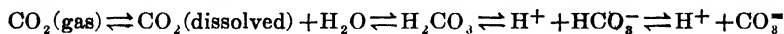
## THEORETICAL.

*Derivation of Equations.*—At the hydron concentration of blood,  $\text{CO}_3^{--}$  and  $\text{PO}_4^{--}$  are negligible and so the equilibria involve only the ions,  $\text{Ca}^{++}$ ,  $\text{H}^+$ ,  $\text{HCO}_3^-$ ,  $\text{HPO}_4^-$ , and  $\text{H}_2\text{PO}_4^-$ , and the molecules,  $\text{CO}_2$ ,  $\text{H}_2\text{CO}_3$ ,  $\text{Ca}(\text{HCO}_3)_2$ ,  $\text{CaHPO}_4$ , and  $\text{Ca}(\text{H}_2\text{PO}_4)_2$ . Such a system may be evolved theoretically by adding to water in

\* Aided by a grant from the Loomis Fund

sequence: (a) carbon dioxide, (b) calcium carbonate, and (c) disodium phosphate.

(a) Carbon dioxide gas dissolved in water yields carbonic acid and its products of dissociation.



Applying the law of mass action and denoting the molecular and ionic concentrations by enclosing their formulas within brackets, the following equations are obtained.

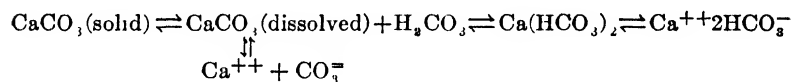
$$K_0 [\text{CO}_2] = [\text{H}_2\text{CO}_3] \quad (1)$$

$$K_1 [\text{H}_2\text{CO}_3] = [\text{H}^+] [\text{HCO}_3^-] \quad (2)$$

$$K_2 [\text{HCO}_3^-] = [\text{H}^+] \cdot [\text{CO}_3^{--}] \quad (3)$$

$K_0$ ,  $K_1$ , and  $K_2$  are the equilibrium constants of carbon dioxide in water.

(b) Calcium carbonate introduced into a solution containing carbonic acid causes the following additional equilibria.



Applying the laws of mass action we obtain

$$[\text{Ca}^{++}] \cdot [\text{CO}_3^{--}] = K_3 \quad (4)$$

and

$$K_4 [\text{H}_2\text{CO}_3] = [\text{Ca}^{++}] \cdot [\text{HCO}_3^-]^2 \quad (5)$$

Equation (5) may likewise be derived by dividing equation (3) into the product of equations (2) and (4) so that  $K_4 = \frac{K_1 K_3}{K_2}$ .

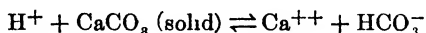
The desired relationship between  $\text{Ca}^{++}$ ,  $\text{H}^+$ , and  $\text{HCO}_3^-$  follows at once by dividing either equations (5) by (2) or (4) by (3).

$$\frac{[\text{Ca}^{++}] \cdot [\text{HCO}_3^-]}{[\text{H}^+]} = \frac{K_4}{K_1} = \frac{K_3}{K_2} = K_5 \quad (6)$$

or

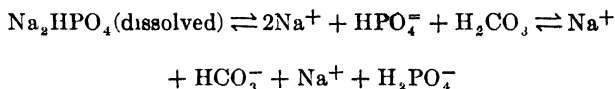
$$[\text{Ca}^{++}] = K_5 \cdot \frac{[\text{H}^+]}{[\text{HCO}_3^-]} \quad (6a)$$

As a matter of fact equation (6) follows at sight by applying the mass action law to the very evident equation:



The lengthy development, however, has been presented so that the equilibrium constants could be calculated from others that are defined in the literature.

(c) Disodium phosphate introduced into the aqueous solution of carbonic acid produces additional equilibria.



It follows then that

$$\frac{[\text{H}_2\text{CO}_3] \cdot [\text{HPO}_4^{=}] }{[\text{HCO}_3^-] \cdot [\text{H}_2\text{PO}_4^-]} = K_6 \quad (7)$$

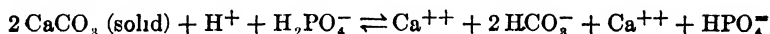
Replacing  $[\text{H}_2\text{CO}_3]$  by its equivalent from equation (2) we get the equilibrium constant for secondary phosphate.

$$\frac{[\text{H}^+] \cdot [\text{HPO}_4^{=}] }{[\text{H}_2\text{PO}_4^-]} = K_1 \cdot K_6 = K_7 \quad (8)$$

Multiplying equations (5), (6), and (7) we obtain

$$\frac{[\text{Ca}^{++}]^2 \cdot [\text{HCO}_3^-]^2 \cdot [\text{HPO}_4^{=}] }{[\text{H}^+] \cdot [\text{H}_2\text{PO}_4^-]} = K_4 \cdot K_5 \cdot K_6 = \left(\frac{K_3}{K_2}\right)^2 \cdot K_7 = (K_5)^2 \cdot K_7 = K_8 \quad (9)$$

Also this equation follows directly by inspection from a system of reacting components expressed by the equation:



This equation is expressed in terms of the ratio of phosphates. For systems in which the total phosphate concentration varies, further relationships must be derived. From equation (8) it follows that

$$\frac{[\text{HPO}_4^{=}] }{[\text{H}_2\text{PO}_4^-]} = \frac{K_7}{[\text{H}^+]} \quad (10)$$

and

$$\frac{[\text{HPO}_4^-] + [\text{H}_2\text{PO}_4^-]}{[\text{HPO}_4^-]} = \frac{K_7 + [\text{H}^+]}{K_7} \quad (11)$$

Dividing through equation (11) by  $[\text{Ca}^{++}]$  we obtain

$$\frac{[\text{HPO}_4^-] + [\text{H}_2\text{PO}_4^-]}{[\text{Ca}^{++}] [\text{HPO}_4^-]} = \frac{K_7 + [\text{H}^+]}{K_7 \cdot [\text{Ca}^{++}]} \quad (11 \text{ a})$$

Substituting the solubility product constant

$$[\text{Ca}^{++}] [\text{HPO}_4^-] = K_9 \quad (12)$$

and solving the equation for total ionic phosphate, we obtain

$$[\text{HPO}_4^-] + [\text{H}_2\text{PO}_4^-] = \frac{K_9}{[\text{Ca}^{++}]} \left( 1 + \frac{[\text{H}^+]}{K_7} \right) \quad (13)$$

or

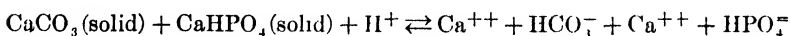
$$[\text{Ca}^{++}] = \frac{K_9}{[\text{HPO}_4^-] + [\text{H}_2\text{PO}_4^-]} \left( 1 + \frac{[\text{H}^+]}{K_7} \right)$$

This equation expresses the equilibrium in terms of the sum of the primary and secondary phosphate ions and serves as an independent method of arriving at the solubility product constant of  $\text{CaHPO}_4$ . However, the ratio of the phosphates is a function of the hydron concentration, and a known value of one phosphate determines the amount of the other. Therefore, the secondary phosphate, which constitutes about 85 per cent of the total phosphates under physiological conditions, expresses simpler relationship with the ions in the system.

Multiplying equation (6) by (12) we obtain

$$\frac{[\text{Ca}^{++}]^2 [\text{HCO}_3^-] \cdot [\text{HPO}_4^-]}{[\text{H}^+]} = K, \quad K_1 = \left( \frac{K_1}{K_2} \right) K_9 = \left( \frac{K_4}{K_1} \right) K_9 = K_{10} \quad (14)$$

This relation follows directly by inspection from a system of reacting components expressed by the equation



Systems studied<sup>1</sup> under conditions in which the tertiary phosphate ion concentration is significant, may be expressed by the following relations.

Multiplying

$$[\text{Ca}^{++}]^3 [\text{PO}_4^{\equiv}]^3 = K_{11} \quad (15)$$

which represents the solubility product of  $\text{Ca}_3(\text{PO}_4)_2$ , by equation (14) we obtain

$$\frac{[\text{Ca}^{++}]^3 [\text{HCO}_3^-] [\text{HPO}_4^-] [\text{PO}_4^{\equiv}]^2}{[\text{H}^+]} = K_5 \cdot K_9 \cdot K_{11} \quad (16)$$

Since the solubility product constant is as yet not accurately known, we may eliminate this constant by utilizing the values of the third dissociation constant of  $\text{H}_3\text{PO}_4$ . This value may be written

$$\frac{[\text{H}^+] [\text{PO}_4^{\equiv}]}{[\text{HPO}_4^-]} = K_{12} \quad (17)$$

Multiplying the square of equation (14) by equation (17) we obtain

$$\frac{[\text{Ca}^{++}]^4 [\text{HCO}_3^-]^2 [\text{HPO}_4^-] [\text{PO}_4^{\equiv}]}{[\text{H}^+]} = (K_5 \cdot K_9)^2 \cdot K_{12} = K_{13} \quad (18)$$

Systems in which the  $\text{PO}_4^{\equiv}$  concentration is significant likewise contain  $\text{CO}_3^{\equiv}$  in appreciable amounts. The equation expressing all the ions under consideration follows by multiplying equation (4) by (18).

$$\frac{[\text{Ca}^{++}]^4 \cdot [\text{HCO}_3^-]^2 \cdot [\text{CO}_3^{\equiv}] [\text{HPO}_4^-] \cdot [\text{PO}_4^{\equiv}]}{[\text{H}^+]} = K_8 K_{13} = K_{14} \quad (19)$$

These equilibrium equations for the ionic components of the carbonates and phosphates of calcium must hold no matter what other coexistent species—aggregated, associated, molecular, or ionic—may be present.

<sup>1</sup> At the suggestion of Dr. Holt who is determining the solubility product constant of the tertiary calcium phosphate we have developed the equilibrium relations for these ions coexisting with  $\text{PO}_4^{\equiv}$ .

## EXPERIMENTAL.

*Method of Procedure.*—The various equilibrium constants, governing the relations among the coexisting ions in question, were evaluated from experimental data. Calibrated tonometers were three times evacuated, filled with hydrogen, and again evacuated. According to the technique of Austin and coworkers (2) definite volumes of the necessary solutions and calculated amounts of  $\text{CO}_2$  were added. The tonometers were finally filled with hydrogen at atmospheric pressure and rotated at least an hour in the electrically regulated water bath at  $38 \pm 0.02^\circ$  to attain equilibrium. Samples of the gas phase were taken out of some of these tonometers and  $\text{CO}_2$  determinations made to check the calculations. Samples of the contained liquid phase were transferred under oil into centrifuge tubes, and after centrifugation analyses were made immediately for total  $\text{CO}_2$ , total calcium, total phosphate, secondary phosphate, and hydron concentration.

*Methods of Determination of the Components*—The *hydron concentration* was determined electrometrically. The apparatus consisted of a Leeds and Northrup direct reading potentiometer of low resistance; enclosed lamp and scale galvanometer, a Weston standard cell certified by the Bureau of Standards with a voltage of 1.01896 serving as a basis for all the electrical measurements, saturated KCl-calomel and hydrogen cells and electrodes and platinized platinum electrodes. The measurements were standardized daily by a 0.05 N KH phthalate solution (3). Hydrogen was passed through the cell previous to the introduction of a sample from the tonometer. The hydrogen cell was rocked until constant equilibrium values were obtained and the molar concentration of hydron read directly from a plotted curve which related the EMF to the CH.

*Total Calcium*—1 cc. of the centrifuged sample under oil was pipetted into a 15 cc. centrifuge tube and determined according to the method of Kramer and Tisdall (4) which one of the authors (5) has shown to be a safe procedure in the presence of phosphate within the range of the hydron concentration studied.

*Total Carbon Dioxide*—1 cc. of the liquid phase kept under albolene was analyzed for total  $\text{CO}_2$  by the method of Van Slyke and Stadie (6).

*Carbon Dioxide of the Gas Phase*—Samples of the gas phase were analyzed for  $\text{CO}_2$  by the Haldane-Henderson apparatus (7).

*Inorganic Phosphates*—These were determined on 1 cc. of the sample kept under oil by Briggs' modification of the Bell-Doisy method (8).

*Secondary Phosphate*—Secondary phosphate was determined on 1 cc. of the sample kept under oil by a method reported in a separate paper (9).

*Preparation and Analysis of Materials.*—Conductivity water was obtained by redistilling water made alkaline with  $\text{KMnO}_4$  through a block tin condenser and was kept in Pyrex flasks free from  $\text{CO}_2$ .

Sodium bicarbonate was prepared by the method of Auerbach and Pick (10). Test—0.1586 gm. of the salt in conductivity water, titrated with methyl orange as indicator, required  $19.01 \pm 0.1$  cc. of 0.1 N HCl. This is in good agreement with the theoretically required amount, 18.88 cc.

Disodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) was crystallized twice from distilled water by adding an equal volume of 95 per cent alcohol and cooling in ice water. The solution was stirred constantly until crystallization was complete. Two liquid phases appear on addition of alcohol and crystallization takes place at the junction of the two liquid phases. As crystallization proceeds the upper phase disappears, leaving but one phase when precipitation is complete. The crystals were filtered on a Buchner funnel with suction, washed with alcohol, and dried under 20 to 30 mm. of pressure at  $100^\circ$ , thus yielding the pure product  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (11).

Calcium carbonate was prepared by bubbling  $\text{CO}_2$  into a concentrated solution of calcium hydroxide at room temperature and washing and drying the resulting precipitate. Stable calcite was thus obtained. The carbonate was added to conductivity water free from  $\text{CO}_2$  in a Pyrex flask connected to a  $\text{CO}_2$ -free burette. A current of  $\text{CO}_2$ -free air was then drawn through the solution to remove the last traces of  $\text{CO}_2$ .

Secondary calcium phosphate ( $\text{CaHPO}_4$ ) was prepared by adding gradually a solution of disodium phosphate to an excess of a solution of calcium chloride and washing the resultant precipitate with calcium chloride and finally with distilled water. The solubility product constant of  $\text{CaHPO}_4$  at  $38^\circ\text{C}$ . will be reported in a separate paper (12).

Mercurous chloride was prepared electrolytically according to the method of Lipscomb and Hulett (13) from redistilled mercury and constant boiling 1.0 N HCl, prepared according to the method of Hulett and Bonner (14). The calomel obtained, heavily laden with finely divided mercury, was washed free from acid with conductivity water and then with saturated KCl. Portions of this calomel were added to solutions of saturated KCl for mutual saturation and the clear solution was decanted into the calomel vessel.

*Mercury*—Redistilled mercury was first shaken in a separatory funnel with a 10 per cent solution of mercurous nitrate, acidified with nitric acid, and then sprayed into a long column of the same solution and finally redistilled by Hulett's method (15).

*Experimental and Calculated Data*—The methods of calculation of the factors involved in the equilibrium equations are presented to interpret the tables given below.



TABLE I

*System: Calcium Carbonate-Calcium Bicarbonate-Carbon Dioxide.*

P <sub>CO<sub>2</sub></sub>	Total CO <sub>2</sub>	Free CO <sub>2</sub> [H <sub>2</sub> CO <sub>3</sub> ]	Total Ca = total Ca(HCO <sub>3</sub> ) <sub>2</sub>	[Ca <sup>++</sup> ] = 1/2[HCO <sub>3</sub> <sup>-</sup> ]	H <sup>+</sup>	K <sub>1</sub> 10 <sup>8</sup>	K <sub>2</sub>
<i>atmosphere</i>	<i>mols/l</i>	$\frac{\alpha_{CO_2}}{22.4} P_{CO_2}$	<i>mols/l</i>	<i>mols/l</i>	<i>mols/l</i>		
0.0263	0.0053	0.00068	0.0023	0.00200	0.50 · 10 <sup>-7</sup>	4.71	160
0.0526	0.0071	0.00136	0.0029	0.00250	(0.48 · 10 <sup>-7</sup> )	4.61	(260)
0.0790	0.0086	0.00208	0.0033	0.00280	1.11 · 10 <sup>-7</sup>	4.22	142
0.1050	0.0099	0.00278	0.0036	0.00300	1.32 · 10 <sup>-7</sup>	3.89	136
0.0263	0.0048	0.00066		0.00180	0.46 · 10 <sup>-7</sup>	3.55	141
0.0523	0.0075	0.00136	0.0031	0.00255	0.71 · 10 <sup>-7</sup>	4.88	181
0.0785	0.0086	0.00208	0.0033	0.00275	1.14 · 10 <sup>-7</sup>	4.00	133
0.1005	0.0108	0.00278	0.0039	0.00330	1.45 · 10 <sup>-7</sup>	5.17	150

$P_{CO_2}$  is the partial pressure of CO<sub>2</sub>, either measured or calculated, expressed in atmospheres

Total CO<sub>2</sub> is the volume of CO<sub>2</sub> measured in the Van Slyke apparatus at the recorded temperature and pressure, reduced to standard conditions.

$\alpha_{CO_2}$ , the absorption coefficient of CO<sub>2</sub>, is the ratio of the volume of CO<sub>2</sub> dissolved (reduced to 0°) to the volume of the water. The value of this ratio for 38° used throughout this work is 0.58 (16). This ratio is, in accordance with Henry's law, independent of the pressure under the conditions of the experiments.  $\frac{\alpha_{CO_2}}{22.4}$  is the value in mols per liter of CO<sub>2</sub> dissolved in pure water. Salts cause a change in the solubility of CO<sub>2</sub> and hence in  $\alpha_{CO_2}$ . The coefficients given were read from curves plotted from Bohr's data (17) for NaCl solutions, with the assumption that the coefficients of CO<sub>2</sub> in our systems are the same as in NaCl of the same equivalent concentrations. The value of  $\frac{\alpha_{CO_2}}{22.4}$  at 38° is 0.026.

$[H_2CO_3] = \frac{\alpha_{CO_2}}{22.4} P_{CO_2}$  is the molal concentration of the dissolved CO<sub>2</sub>. H<sub>2</sub>CO<sub>3</sub> calculated in this way involves the assumption that CO<sub>2</sub> does not exist as such in solution but hydrated as CO<sub>2</sub> · H<sub>2</sub>O or H<sub>2</sub>CO<sub>3</sub>, a fact substantiated by the present work.

Total Ca(HCO<sub>3</sub>)<sub>2</sub> expressed in mols per liter, equals (a) total Ca in mols per liter, or  $\frac{\text{total } [CO_2] - [H_2CO_3]}{2}$  where calcium bicarbonate is the calcium component, (b)  $\frac{\text{total } [CO_2] - ([NaHCO_3] + [H_2CO_3])}{2}$  where NaHCO<sub>3</sub> is also present; (c) total [Ca] - ([CaHPO<sub>4</sub>] + [Ca(H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub>]) where phosphates are present.

TABLE  
*System Calcium Carbonate-Calcium Bicarbonate-Sodium Bicarbonate-Carbon Dioxide.*

P <sub>CO<sub>2</sub></sub>	Total CO <sub>2</sub>	Free CO <sub>2</sub> [H <sub>2</sub> CO <sub>3</sub> ]	Total Ca = total Ca(HCO <sub>3</sub> ) <sub>2</sub>	Ca <sup>++</sup> from Ca(HCO <sub>3</sub> ) <sub>2</sub> = ½(HCO <sub>3</sub> ) <sub>2</sub>	Concen- tration of NaHCO <sub>3</sub>	HCO <sub>3</sub> <sup>-</sup> from NaHCO <sub>3</sub>	Total HCO <sub>3</sub> <sup>-</sup>	H <sup>+</sup>	K <sub>1</sub> 10 <sup>3</sup>	K <sub>2</sub>
	<i>mols/l</i>	$\alpha_{\text{CO}_2} \frac{P_{\text{CO}_2}}{22.4}$	<i>mols l</i>	<i>mols/l</i>	<i>mols.l</i>	<i>mols, l</i>	<i>mols l</i>	<i>mols/l</i>		
<i>atmosphere</i>										
0.0263	0.0160	0.00068	0.00024	0.00019	0.0150	0.01170	0.01208	0.17·10 <sup>-7</sup>	4.09	136
0.0526	0.0169	0.00136	0.00035	0.00030	0.0150	0.01170	0.01201	0.31·10 <sup>-7</sup>	3.25	116
0.0790	0.0180	0.00208	0.00060	0.00051	0.0150	0.01170	0.01272	0.53·10 <sup>-7</sup>	3.96	122
0.1050	0.0190	0.00278	0.00069	0.00058	0.0150	0.01170	0.01286	0.73·10 <sup>-7</sup>	3.46	103
0.0263	0.0077	0.00066	0.00059	0.00050	0.0075	0.00608	0.00708	0.23·10 <sup>-7</sup>	3.80	154
0.0526		0.00136	0.00114	0.00101	0.0075	0.00608	0.00810		4.87	
0.0790	0.0111	0.00208	0.00170	0.00148	0.0075	0.00608	0.00904	0.99·10 <sup>-7</sup>	5.82	135

$\gamma_1 \cdot Ca(HCO_3)_2$  is the degree of dissociation obtained from the experimentally determined values for the corresponding solutions of  $Ca(H_2C_2O_4)_2$  (18). The validity of such procedure follows from Noyes' rule "that the degree of ionization of a salt in a mixture is practically equal to that of a salt of the same valence type at the same total ion concentration." The values of  $\gamma$  ranged from 0.82 to 0.92 as read from curves plotted for the systems under consideration.

$\gamma_2 NaHCO_3$  is 0.78 for 0.015 M and 0.81 for 0.0075 M  $NaHCO_3$ .

$\gamma_3 CaHPO_4$  is 0.62 to 0.66 for the given concentrations as calculated from conductivity data (19)

$\frac{1}{2}[H_2PO_4^-]$  in mols per liter is the difference between the total measured phosphorus and the measured molal  $HPO_4$

TABLE III—System: Phosphates and

Pco <sub>2</sub>	Total CO <sub>2</sub>	Free CO <sub>2</sub> [H <sub>2</sub> CO <sub>3</sub> ]	Total Ca	Total P	Bicarbonates			
					[Ca <sup>++</sup> ] = $\frac{1}{2}[HCO_3^-]$	Ca(HCO <sub>3</sub> ) <sub>2</sub>	NaHCO <sub>3</sub> added	HCO <sub>3</sub> <sup>-</sup> from NaHCO <sub>3</sub>
atmo- sphere	mols/l	$\frac{\alpha CO_2}{22.4} Pco_2$	mols/l	mols/l	mols/l	mols/l	mols/l	mols/l
0.0230	0.00430	0.00068	0.00235	0.00064	0.00160	0.00174		
0.0526	0.00565	0.00136	0.00262	0.00050	0.00195	0.00214		
0.0725	0.00630	0.00191	0.00270	0.00046	0.00200	0.00220		
0.0790	0.00699	0.00205	0.00285	0.00044	0.00220	0.00244		
0.0945	0.01278	0.00246	0.00200	0.00069	0.00126	0.00137	0.0075	0.00608
0.0989	0.01305	0.00264	0.00215	0.00081	0.00132	0.00143	0.0075	0.00608
0.1121	0.01385	0.00303	0.00232	0.00078	0.00151	0.00164	0.0075	0.00608
0.1410	0.01520	0.00381	0.00250	0.00064	0.00176	0.00193	0.0075	0.00608

$\frac{[HPO_4^-]}{[H_2PO_4^-]}$  "determined," is obtained directly from the given measured values and this ratio calculated follows from equation (9) by substituting appropriate values for the hydron concentration

*Estimation of the Average Error*—The average errors in analysis of the components of the liquid phase were approximately within the following ranges: 1 per cent for the total CO<sub>2</sub>, 1 per cent for the hydron concentration; 4 per cent for HPO<sub>4</sub>, 4 per cent for total P; and 4 per cent for total Ca. Analysis of the gas phase at the end of saturation by the Haldane-Henderson apparatus is accurate to about 0.1 vol. per cent. The corresponding tension at 38° and 760 mm. is  $\frac{0.10}{100} (760-49) = 0.7$  mm. of Hg. The average difference of tensions calculated and analyzed was within 1 mm.

We may calculate the degree of uncertainty of the equilibrium constant for the complete equation (14). If  $d(\text{Ca})$  is an error in  $[\text{Ca}^{++}]$ , then the error in  $K$  is  $\frac{\delta K}{\delta(\text{Ca})} \cdot d(\text{Ca})$ , and similarly for each of the other factors. The general equation from which the average range of error may be calculated is derived by (a) converting the equation into logarithmic form since  $\frac{dK}{K} = d(\log K)$ , (b) partially differentiating with respect to each of the components; and (c) summing the differentials, squaring, and solving for  $\frac{dK}{K}$  which on substitution yields an average range of error of  $\pm 10$  per cent.

*Calcium at Various Carbon Dioxide Tensions*

Phosphates				Total Ca <sup>++</sup>	H <sup>+</sup>	K <sub>4</sub> 10 <sup>8</sup>	K <sub>5</sub>	K <sub>6</sub> 10 <sup>8</sup>		K <sub>8</sub> 10 <sup>8</sup>	K <sub>10</sub> 10 <sup>8</sup>
[Ca <sup>++</sup> ] = [HPO <sub>4</sub> <sup>-</sup> ]	[Ca <sup>++</sup> ] = ½[H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> ]	[HPO <sub>4</sub> <sup>-</sup> ]/[H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> ]						[Ca <sup>++</sup> ]/[HPO <sub>4</sub> <sup>-</sup> ]	From equation (12)		
		Deter- mined	Calcu- lated								
mols/l	mols/l			mols/l	mols/l						
0.00036	0.000043	4.7	4.5	0.00200	0.53 · 10 <sup>-7</sup>	3.01	120	72	73	3.63	4.45
0.00028	0.000033	4.2	4.1	0.00226	0.58 · 10 <sup>-7</sup>	2.53	152	70	63	5.62	9.63
0.00025	0.000040	2.8	2.6	0.00229	0.90 · 10 <sup>-7</sup>	2.00	102	58	55	2.92	5.83
0.00023	0.000045	2.7	2.5	0.00248	0.96 · 10 <sup>-7</sup>	2.35	114	90	57	3.35	6.48
0.00034	0.000080	2.1	2.0	0.00168	1.20 · 10 <sup>-7</sup>	5.05	121	61	70	3.66	6.88
0.00040	0.000100	2.0	1.9	0.00182	1.25 · 10 <sup>-7</sup>	5.28	128	80	72	4.06	9.78
0.00038	0.000100	1.8	1.7	0.00199	1.40 · 10 <sup>-7</sup>	5.44	130	65	75	4.22	9.28
0.00030	0.000090	1.7	1.5	0.00215	1.60 · 10 <sup>-7</sup>	5.20	129	75	62	4.53	8.32

*Results.*—Three series of experiments were done on systems equilibrated in tonometers at  $38^\circ$  at  $\text{CO}_2$  tensions varying from 17 to 110 mm., according to the procedure outlined above. The first series of eight experiments was made on the system,  $\text{CaCO}_3$ — $\text{Ca}(\text{HCO}_3)_2$ — $\text{CO}_2$  (Table I). The second series of seven experiments was done as above with the addition of 0.0075 and 0.015 M  $\text{NaHCO}_3$  (Table II). The third series of eight experiments was carried out on systems which contained, in addition to  $\text{CaCO}_3$ ,  $\text{Ca}(\text{HCO}_3)_2$ ,  $\text{NaHCO}_3$ , and  $\text{CO}_2$ , the components  $\text{CaHPO}_4$  and  $\text{Ca}(\text{H}_2\text{PO}_4)_2$  (Table III). The values of the equilibrium constants  $K_4$  and  $K_5$  are derived for each experiment in Tables I, II,

and III; K<sub>8</sub>, K<sub>9</sub>, and K<sub>10</sub> are obtained from the data in Table III. The average values of the equilibrium constants are summarized in Table IV.

TABLE IV

*Average Values of the Equilibrium Constants from the Experimental Data*

	K <sub>1</sub> 10 <sup>5</sup>	K <sub>2</sub>	K <sub>9</sub> 10 <sup>5</sup>	K <sub>8</sub> 10 <sup>5</sup>	K <sub>10</sub> 10 <sup>5</sup>
Values . .	4 14±0 14	133±3	67±7	4±0 4	7 6±0 6
No. of determinations	23	21	20	8	8

#### DISCUSSION OF EQUILIBRIUM CONSTANTS.

Some of the equilibrium constants involved are known accurately and others may be interpolated. These may be compared with the values experimentally obtained.

K<sub>1</sub>, the first dissociation constant of carbonic acid, is the true dissociation constant of carbonic acid and is to be distinguished from the apparent constant, for the concentration of carbonic acid is really the sum of carbonic acid and its anhydride and the apparent dissociation constant K<sub>1</sub> is given by the following equation.

$$K_1 ([H_2CO_3] + [CO_2]) = [H^+] [HCO_3^-]$$

Walker and Cormack (20) found K<sub>1</sub> to be  $3.04 \times 10^{-7}$  at 18° and calculated it to be  $3.4 \times 10^{-7}$  at 25°. L. J. Henderson found it to be  $4.2 \times 10^{-7}$  at 38° (21). The true dissociation constant is  $\frac{K'_1}{n}$  where  $n$  is the fractional amount of the total CO<sub>2</sub> in solution existing in the form of H<sub>2</sub>CO<sub>3</sub>. Walker and Cormack (20) maintain that  $n$  is always greater than 0.5. We have found  $n$  to approach unity and so have used the value of K'<sub>1</sub> for K<sub>1</sub> in our calculations.

K<sub>2</sub>, the second dissociation constant of carbonic acid, was determined by Seyler and Lloyd (22) as  $4.91 \times 10^{-11}$  at 25°, a better result than had been previously obtained (23,24). Johnston (25) recalculated K<sub>2</sub> at 25° from Walker and Cormack's data and found it to be  $6 \times 10^{-11}$  and  $6.4 \times 10^{-11}$  from Shields' data (26) on the hydrolysis of sodium carbonate, results identical with those computed by McCoy (24), and those obtained by Auerbach and

Pick (10). We have calculated this value by means of the van't Hoff formula as  $7.2 \times 10^{-11}$  at  $38^\circ$ .

$\frac{K_1}{K_2}$ , the ratio of the first and second dissociation constants of carbonic acid, Johnston (25) calculated as 5,600 from McCoy's (24) data on the carbonate-bicarbonate equilibrium at  $25^\circ$ . Using the data given above for  $38^\circ$  the value is 5,800.

$K_3$ , the solubility product constant of calcium carbonate, Leather and Sen (27) found to vary inversely with the temperature, but obtained rather irregular results, the value at  $40^\circ$  approximating  $0.5 \times 10^{-8}$ . Seyler and Lloyd (28) made studies at "laboratory temperature" ( $20^\circ$ ) and obtained  $0.86 \times 10^{-8}$ . They also observed that the constant was not affected by the presence in solution of small amounts of added  $\text{Ca}^{++}$  from other salts, but that the presence of small amounts of neutral salts with no ion in common increased the total calcium in solution. Johnston recalculated the data of Schlöesing (29) and Engel (30) and obtained a concordant value of  $0.98 \times 10^{-8}$  at  $16^\circ$ . Wells (31) determined the solubility product constant at several temperatures with air containing 3.2 parts  $\text{CO}_2$  per 10,000 as the gas phase:

$$0.93 \times 10^{-8} \text{ (20}^\circ\text{)}, 0.87 \times 10^{-8} \text{ (25}^\circ\text{)}, 0.81 \times 10^{-8} \text{ (30}^\circ\text{)}$$

Osaka (32) recalculated the data of McCoy and Smith (33) and obtained the constant  $0.57 \times 10^{-8}$ . The solubility product constant may be calculated from our experimental results at  $38^\circ$  for

$$K_3 = \frac{K_1}{\frac{K_1}{K_2}} = \frac{4.14 \times 10^{-7}}{5,800} = 0.71 \times 10^{-8}$$

a value which compares favorably with the reliable constants presented above.

$$K_4 = \frac{[\text{Ca}^{++}] [\text{HCO}_3^-]^2}{[\text{H}_2\text{CO}_3]} = \frac{K_1 K_2}{K}$$

Rona and Takahashi (34) found experimentally at "laboratory temperature" ( $18^\circ$ ) a value of  $11.6 \times 10^{-5}$ . Johnston's calculations from Schlöesing's data show a general average of  $5.3 \times 10^{-5}$  ( $16^\circ$ ) from Engel's data  $5.47 \times 10^{-5}$  ( $16^\circ$ ) and from Seyler and Lloyd's data  $4.80 \times 10^{-5}$  ( $20^\circ$ ). The value of  $K_4$  from our experi-

mental data at 38° shows a general average, from twenty-two determinations, of  $4.14 \pm 0.14 \times 10^{-5}$ .

$$K_5 = \frac{[Ca^{++}] \cdot [HCO_3^-]}{[H^+]} = \frac{K_4}{K_1} = \frac{K_8}{K_2}$$

Rona and Takahashi (34), a decade ago, found experimentally that the value for this constant was 350 at 18°. In their work the CO<sub>2</sub> phase was not adequately controlled and so affected the hydron concentration. The analyzed Ca(HCO<sub>3</sub>)<sub>2</sub> was assumed completely ionized and some of the equilibrium constants used in their calculations have since been revised. Calculation of K<sub>5</sub> from the ratio K<sub>4</sub>/K<sub>1</sub> gives 111 and from K<sub>3</sub>/K<sub>2</sub> also 111. From our experimental results at 38° the general average of twenty-two determinations for this equilibrium constant is  $133 \pm 3$ . The deviation of the calculated from the experimental values is 20 per cent, a value greater than the calculated percentage error and hence must be attributed to unknown experimental error or to the value of the constants used though they are the most accurate known.

$$K_6 = \frac{[H_2CO_3] [HPO_4^-]}{[HCO_3^-] [H_2PO_4^-]} = \frac{K_7}{K_1}$$

Since Abbott and Bray (35) found K<sub>7</sub>, the second dissociation constant of phosphoric acid to be  $2 \times 10^{-7}$  at 25° and  $2.4 \times 10^{-7}$  at 38°, K<sub>6</sub> becomes 0.57.

$$K_8 = \frac{[Ca^{++}]^2 \cdot [HCO_3^-]^2 \cdot [HPO_4^-]}{[H^+] \cdot [H_2PO_4^-]} = K_4 \cdot K_7, \quad K_6 = \left(\frac{K_8}{K_7}\right) \cdot K_7 = (K_6)^2 \cdot K_7$$

The calculated value of K<sub>8</sub> is  $3.0 \times 10^{-3}$ . The general average of K<sub>8</sub> calculated from the experimental data given above is  $4.0 \pm 0.4 \times 10^{-3}$ .

$$K_9 = [Ca^{++}] [HPO_4^-]$$

Values of this solubility product constant found in the literature are conflicting. The concentration of Ca<sup>++</sup> and HPO<sub>4</sub><sup>-</sup> for the aqueous systems in this work, in equilibrium with solid CaHPO<sub>4</sub> among other components at various CO<sub>2</sub> tensions, yields  $71 \times 10^{-8}$  as the average value for this constant at 38°. Calculation of the constant from equation (12) gives a general average of  $66 \times 10^{-8}$ .

$$K_{10} = \frac{[Ca^{++}]^2 [HCO_3^-] [HPO_4^-]}{[H^+]} = K_5 \cdot K_9 = \left(\frac{K_8}{K_2}\right) K_9 = \left(\frac{K_4}{K_1}\right) K_9$$

Freudenberg and György (36) modified the equation of Rona and Takahashi (34)

$$\frac{[Ca^{++}] \cdot [HCO_3^-]}{[H^+]} = K_1$$

into the empirical form

$$\frac{[Ca^{++}]}{[H^+]} \frac{[HCO_3^-] \cdot [HPO_4^-]}{[H^+]} = K_8$$

This equation has neither theoretical nor experimental basis, and the values of  $Ca^{++}$  calculated from it are not concordant with our experimental data. The value of  $K_{10}$  calculated from the product  $K_5 \cdot K_9$  or its equivalents above is  $8.8 \times 10^{-5}$ . The general average of this constant calculated from the experimental data is  $7.6 \pm 0.6 \times 10^{-5}$ .

The application of this equation to determine the calcion concentration in normal and pathological conditions will be reported in a subsequent paper.

$$K_{11} = [Ca^{++}]^3 [PO_4^{=}]^2$$

This solubility product constant has as yet not been accurately determined. Calculated from solubility values at "laboratory temperature" obtained in the literature one of the authors found  $K_{11}$  to be  $2.8 \times 10^{-23}$  at  $18^\circ$  (5).

$$K_{12} = \frac{[H^+] \cdot [PO_4^{=}]}{[HPO_4^-]}$$

Values for this tertiary dissociation constant range from 3.6 to  $5.6 \times 10^{-13}$  at  $18^\circ$  (3). The dissociation curve for the phosphates plotted as a function of the hydron concentration, however, indicates this constant to be of an order of  $10^{-12}$ .

$$K_{13} = \frac{[Ca^{++}]^4 [HCO_3^-]^2 \cdot [HPO_4^-] [PO_4^{=}]}{[H^+]} = (K_5 \cdot K_9)^2 \cdot K_{12}$$

The value of this constant depends on the known values of  $K_5$  and  $K_9$  and the undetermined value of  $K_{12}$ . Until accurate data at  $38^\circ$  are available the equation cannot be solved. It seems best not to offer an approximation.

$$K_{14} = \frac{[Ca^{++}]^5 [HCO_3^-]^2 \cdot [CO_3^{=}] [HPO_4^-] [PO_4^{=}]}{[H^+]} = K_8 \cdot K_{13}$$



The value of  $K_3$  is  $0.71 \times 10^{-8}$  at  $38^\circ$ . The value of  $K_{14}$  also depends on the determination of the tertiary dissociation constant at  $38^\circ$ . Because this value is not definitely determined it seems best for the present to use the simpler relationship in which  $\text{CO}_3^{=}$  and  $\text{PO}_4^{=}$  are not involved, namely

$$\frac{[\text{Ca}^{++}]^2 [\text{HCO}_3^-] [\text{HPO}_4^-]}{[\text{H}^+]} = K_{10}$$

#### SUMMARY.

The equilibrium relations for  $\text{Ca}^{++}$ ,  $\text{H}^+$ ,  $\text{HCO}_3^-$ ,  $\text{CO}_3^{=}$ ,  $\text{H}_2\text{PO}_4^-$ ,  $\text{HPO}_4^-$ , and  $\text{PO}_4^{=}$  at  $38^\circ\text{C}$ . have been studied and their constants obtained experimentally are:

$$(1) \quad \frac{[\text{Ca}^{++}] \cdot [\text{HCO}_3^-]^2}{[\text{H}_2\text{CO}_3]} = K_4 = 4.14 \pm 0.14 \times 10^{-5}$$

$$(2) \quad \frac{[\text{Ca}^{++}] \cdot [\text{HCO}_3^-]}{[\text{H}^+]} = K_5 = 133 \pm 3 \text{ or } [\text{Ca}^{++}] = 133 \frac{[\text{H}^+]}{[\text{HCO}_3^-]}$$

$$(3) \quad [\text{Ca}^{++}] \cdot [\text{CO}_3^{=}] = K_3 = 0.71 \pm 0.05 \times 10^{-8}$$

$$(4) \quad [\text{Ca}^{++}] \cdot [\text{HPO}_4^-] = K_9 = 67 \pm 7 \times 10^{-8}$$

$$(5) \quad [\text{HPO}_4^-] + [\text{H}_2\text{PO}_4^-] = \frac{67 \cdot 10^{-8}}{[\text{Ca}^{++}]} \left( 1 + \frac{[\text{H}^+]}{2.4 \cdot 10^{-7}} \right)$$

$$\text{or } [\text{Ca}^{++}] = \frac{67 \cdot 10^{-8}}{[\text{HPO}_4^-] + [\text{H}_2\text{PO}_4^-]} \left( 1 + \frac{[\text{H}^+]}{2.4 \cdot 10^{-7}} \right)$$

$$(6) \quad \frac{[\text{Ca}^{++}]^2 \cdot [\text{HCO}_3^-]^2 [\text{HPO}_4^-]}{[\text{H}^+] \cdot [\text{H}_2\text{PO}_4^-]} = K_8 = 4.0 \pm 0.4 \times 10^{-8}$$

$$(7) \quad \frac{[\text{Ca}^{++}]^2 \cdot [\text{HCO}_3^-] \cdot [\text{HPO}_4^-]}{[\text{H}^+]} = K_{10} = 7.6 \pm 0.6 \times 10^{-5}$$

$$\text{or } [\text{Ca}^{++}] = \sqrt{(7.6 \times 10^{-5}) \frac{[\text{H}^+]}{[\text{HCO}_3^-] \cdot [\text{HPO}_4^-]}}$$

$$(8) \quad \frac{[\text{Ca}^{++}]^4 \cdot [\text{HCO}_3^-]^2 \cdot [\text{HPO}_4^-] [\text{PO}_4^{=}]}{[\text{H}^+]} = K_{13} = (0.8 \times 10^{-8}) \cdot K_{12}$$

$$(9) \quad \frac{[\text{Ca}^{++}]^5 \cdot [\text{HCO}_3^-]^2 \cdot [\text{CO}_3^{=}] [\text{HPO}_4^-] \cdot [\text{PO}_4^{=}]}{[\text{H}^+]} = K_{14} = (0.6 \times 10^{-16}) \cdot K_1$$

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# THE ESTIMATION OF THE HYDROGEN CYANIDE CONTENT OF AMYGDALIN BY THE AERATION METHOD.

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(Received for publication, November 1, 1923 )

In a previous paper<sup>1</sup> the author set forth a new method for estimating simple, soluble cyanides, in which freed hydrogen cyanide is washed by a current of air from one flask containing an acidified cyanogen product into a second flask containing dilute alkali, and the mixture obtained is then titrated with standard silver nitrate solution, using a few drops of potassium iodide as an indicator. Some recent work has shown that this procedure will yield excellent results when applied to the determination of the hydrogen cyanide in amygdalin, the glucoside being hydrolyzed by the enzyme, emulsin. It is the object of this paper to apply this method to the estimation of the hydrogen cyanide content of amygdalin and to suggest that further valuable applications of the aeration procedure may be made to a quantitative study of cyanogenetic plants in general.

## *Procedure.*

Prepare an apparatus suitable for aeration (such as the Folin ammonia aeration apparatus). In one flask place a 0.10 gm. sample of amygdalin, add about 0.05 gm. of emulsin, 100 cc. of water, and a few drops of amyl or capryl alcohol. Insert stopper and close the aeration tubes by means of rubber tubing and pinch-cocks. Shake thoroughly to mix the enzyme and amygdalin, and warm to 45° for 15 minutes. Connect the flask containing this mixture to a second flask containing 100 to 150 cc. of 5 per cent sodium hydroxide and attach to a suction pump. Now pass a current

<sup>1</sup> Roe, J. H., *J. Am. Chem. Soc.*, 1923, xlv, 1878.

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of air through the flask containing the amygdalin mixture into the flask containing alkali at the rate of about 3 liters per minute. Continue aeration for about 3 hours, then disconnect the flasks, add 10 drops of 10 per cent potassium iodide to the alkaline cyanide solution in the second flask and titrate with 0.01 N silver nitrate until a faint turbidity appears. The reading of the burette multiplied by 0.0005404 will give the number of grams of hydrogen cyanide in the sample of amygdalin. The initial warming at 45° is not necessary if aeration is continued an hour longer, which affords more time for the enzyme to work. The reduced time necessary for aeration and the added assurance of complete recovery make the warming of the mixture desirable.

### DISCUSSION.

The method is significant in two respects: (1) it affords a very accurate and easy procedure for estimating the hydrogen cyanide of amygdalin, and hence for the assay of amygdalin itself, and (2) it opens the possibilities of estimating the hydrogen cyanide content of glucosides in cyanogenetic plants, where enzymic hydrolysis is favorable. It is an important fact *that the enzyme continues to work while aeration is in progress*. In plant estimations where autolysis is the only means of hydrolytic cleavage, the aeration procedure should prove a more favorable and more convenient adaptation than the older distillation methods. Boiling organic mixtures with an acid is an unsatisfactory mechanical procedure, and in the case of amygdalin yields notoriously low recoveries due to a continued hydrolysis of the cyanogen product after the glucoside has been split, which liberates the nitrogen as ammonia. Enzymic hydrolysis, where possible, is therefore indicated. Hence the older distillation methods are undesirable, because poor recoveries are experienced when acids are used as hydrolytic agents and, when an enzyme is used as the hydrolyzing agent, the heating may kill the enzyme before hydrolysis has been completed. In the aeration method, the hydrolysis of the glucoside and the removal of freed hydrogen cyanide are begun at essentially the same time and continued simultaneously until the hydrolysis is completed and all the hydrogen cyanide is carried over into the alkaline receptor.

## SUMMARY.

A method has been devised for estimating the HCN in amygdalin by enzymic hydrolysis and application of the aeration procedure. The method should prove a favorable means for quantitative investigations of cyanogenetic plants in general.



# STUDIES ON THE ABSORPTION OF METALLIC SALTS BY FISH IN THEIR NATURAL HABITAT.

## II. THE ABSORPTION OF NICKEL BY *FUNDULUS HETEROCLITUS*.

By ADRIAN THOMAS.\*

(Received for publication, November 8, 1923.)

Salts of certain metals such as copper and cadmium, when added to sea water, prove to be exceedingly toxic to fish immersed in such solutions. Some other metals apparently have no toxic action in sea water; among which are cobalt, nickel, and manganese. A third class of metals, of which zinc is a member, appears to be only slightly toxic in dilute solutions made in sea water.

In the case of copper the metal is absorbed by the fish and probably forms a complex compound with the proteins since a blue-green coloration is noticed in the tissues of fish that have been subjected to solutions of copper in sea water. White and Thomas<sup>1</sup> found that the amount of copper absorbed increased with the time that the fish were subjected to copper solutions, up to a certain limit which caused death.

Nickel salts behave differently in that they are not toxic. The method of exposure to the solutions was to keep the fish for various periods of time in glass jars containing 6 liters of sea water, to which the desired amount of nickel chloride had been added. These solutions were aerated by passing a constant stream of air through them. Under these conditions *Funduli* lived for weeks without showing any signs of sickness whatever, in spite of the fact that nickel was absorbed.

After removal from the solutions the fish were thoroughly washed and a stream of tap water was passed through the alimentary canal. They were then dried to constant weight at about 105°C. and ground in a mortar. A 10 gm. sample was

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<sup>1</sup> White, G. F., and Thomas, A., *J. Biol. Chem.*, 1912, xi, 381.



taken for analysis, this was ashed in a muffle and the ash dissolved in hydrochloric acid, any free carbon being filtered off. The filtrate was made ammoniacal, a white precipitate of phosphates resulting, ammonium sulfide was added without filtering, and the whole mass warmed. After cooling, sufficient, cold, dilute hydrochloric acid was added to redissolve the phosphates and the remainder, a black precipitate, was filtered off. The whole filter was digested in hot nitrohydrochloric acid to dissolve the nickel sulfide, then diluted and filtered. To the filtrate a little citric acid was added, to prevent precipitation of iron, and finally a 1 per cent solution of dimethylglyoxime in alcohol, after which the solution was made ammoniacal. After warming on the water bath a few hours a precipitate of nickel oxime was obtained which was filtered on alundum cones, dried at a temperature of 100°C., and weighed. This method was tested, and proved satisfactory, by adding a known amount of nickel to some dried untreated fish and then proceeding as above, having first ascertained that no precipitate was obtained with dimethylglyoxime when untreated fish were tested without the addition of nickel.

In Table I the amount of nickel absorbed from solutions of different concentrations in a given time by *Funduli*, is recorded in terms of metallic nickel in the dried material.

The results given in Table I are the averages of analyses of samples from at least two experiments in each of which about ten fish were used. It may be noted that in certain cases the fish apparently contained more nickel after only a few hours exposure than others did after a longer exposure. This cannot be explained at present otherwise than to suggest it might be due to individual variation or that some of the nickel first absorbed was again eliminated. This latter explanation does not seem probable since there is a tendency for absorption to be directly proportional to the time of exposure, and the fish had not been removed from the nickel solutions at any time.

However, when the fish are subjected to solutions of the nickel chloride for some time and then placed in running sea water, they eliminate nickel. A number of *Funduli* were subjected to a N/500 solution of nickel chloride in sea water for 90 hours. At the end of that time some were washed and prepared immediately

for analysis, the remainder were put into running sea water for 8 days after which time they were prepared for analysis. Considerable nickel could be detected in the fish that were not placed in running water, while those that were, appeared to be practically free from nickel after the 8 days. This experiment was duplicated. In the case of fish subjected to copper solutions and then put in running sea water the copper was retained for some time as has been stated in a previous paper.<sup>2</sup>

Though apparently non-toxic in sea water, nickel salts appear to be very toxic in solutions of fresh water. A number of fish of the same species (*Fundulus heteroclitus*) were found in a land-

TABLE I  
*Nickel Absorbed from Solutions by Funduli*

Concentration of solutions	Time	Ni	Concentration of solutions	Time	Ni
N	hrs	per cent	N	hrs	per cent
0 016	48	0 0079	0 001	24	0 0029
0 004	1	0 0050	0 001	48	0 0019
0 004	6	0 0050	0 001	168	0 0017
0 004	35	0 0080	0 0005	24	0 0022
0 004	168	0 0090	0 0005	48	0 0026
0 002	3	0 0014	0 0005	168	0 0010
0 002	4	0 0028	0 0005	336	0 0024
0 002	48	0 0012	0 00025	168	0 0013
0 002	168	0 0024	0 00025	336	0 0022
0 002	336	0 0044	0 000125	168	0 0018

locked pond which contained water having a density of only 1.0008. These fish lived well in fresh water used as a control, but if enough nickel chloride was added to the water to give a concentration of N/8,000 the fish died within a few hours. Under conditions of the experiments those fish kept in jars of pure tap water as controls survived at least 12 hours, but if kept in running tap water they could be kept quite a while. When enough sugar was added to fresh water to make a M/50 solution, life could be considerably prolonged, yet if enough nickel chloride was added to the sugar solution to make a concentration of N/1,000 the fish died in a very short time. The increase in os-

<sup>2</sup> Thomas, A, *Tr. Am. Fisheries Soc.*, 1915, xlv, 120.

motie pressure by means of sugar seemed to exert no influence when the nickel was present.

When fish are poisoned by the metallic salts they show distinct signs of intoxication which are manifest by their movements and general appearance which are unlike those shown when death is caused simply by the change from salt to fresh water. Although fish were poisoned by N/8,000 solutions of nickel chloride in fresh water, they exhibited no signs of sickness even after having been subjected to solutions of N/250 concentration in sea water for 2 weeks.

From the data at hand it may be concluded that *Funduli* absorb considerable nickel from solutions of the chloride in sea water without evidence of intoxication. In fresh water, however, nickel chloride is toxic even though the fish are accustomed to fresh water. Nickel was eliminated from *Funduli* after the latter had been in running sea water for 8 days subsequent to their immersion in a solution of nickel chloride.

## A MODIFICATION OF ISAACS' COLORIMETRIC DETERMINATION OF BLOOD CHLORIDES.

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(Received for publication, November 19, 1923)

Isaacs (1922) has described a colorimetric method for the determination of the chlorides of the blood. The method is based on the fact that silver chromate is more soluble than silver chloride and dissolves in a solution of chlorides, the silver being reprecipitated as the chloride, leaving the chromate radicle in solution as alkali chromate. In the determination an excess of silver chromate is used with blood filtrate and when the reaction is complete the remaining silver chromate and most of the silver chloride are removed by centrifugation and filtration. The turbidity, due to unremoved silver chloride, is discharged by the addition of dilute ammonium hydroxide and the alkali chromate estimated colorimetrically against a standard chromate solution.

The light yellow shade of the alkali chromate is very difficult to match accurately in the colorimeter. Isaacs recommends viewing the color through a blue glass as suggested by Michaelis (1921) to overcome this difficulty. Also the method requires 10 cc. of blood filtrate, prepared by the technique of Folin and Wu (1919), to give a color of sufficient intensity.

The writer, even with the use of a blue glass, has been unable to match the chromate colors accurately, and has developed a new colorimetric reaction for use with this method.

Several attempts were made to devise a method for making the color comparisons in acid instead of alkaline solution. This would allow the use of the darker and more intense chromic acid color. However, no satisfactory method of avoiding turbidity in the acid solution was found.

Later, the reducing action of potassium iodide on chromic acid in acid solution was tried. In this reaction chromium is

reduced from the hexavalent state to the trivalent state and free iodine is released, with the production of the familiar red-brown color of iodine solutions. This color is of a shade readily matched with accuracy in the colorimeter and of an intensity such that 5 cc. of blood filtrate, representing 0.5 cc. of blood, are amply sufficient for a test.

The colors produced by solutions of different concentrations were checked in the colorimeter and found to be proportional over a wide range of concentration. The chromium sulfate formed in the reduction is greenish in color, but is so faint in comparison with the intense iodine color that the latter is not materially modified. The reduced chromium is proportional to concentration so no error is introduced by it.

The turbidity due to unremoved silver chloride in blood filtrates, treated with silver chromate by the Isaacs method, is entirely discharged by the excess of potassium iodide used in the above reaction.

Ten blood filtrates were tried with potassium iodide and sulfuric acid in the absence of chromates and found not to release free iodine.

An iodine solution of approximately twice the color intensity used in the new method and containing proportional amounts of potassium iodide and sulfuric acid was diluted 1:1 with distilled water and also with each of ten blood filtrates. The one diluted with distilled water was used as standard and the others were compared with it in the colorimeter at once and at half hour intervals. No fading of the iodine color by any of the blood filtrates was observed after 2 hours.

A new method, retaining Isaacs' procedure of using silver chromate, but developing the iodine color as described, is given here.

#### *Reagents Required.*

##### *1. Silver Chromate.*—Prepared as described by Isaacs (1922).

“ . . . adding slowly 200 cc of a 5.5 per cent solution of potassium chromate to 100 cc. of a boiling solution of silver nitrate (10 per cent). The silver chromate settles out rapidly. Drops of the chromate solution are added until there is a slight excess of chromate, which gives the solution a yellow color. After cooling, the silver chromate is thoroughly washed with distilled water and finally air-dried on a Buchner funnel.”

The boiling temperature should be adhered to, as an excess of chromate in the cold forms some silver bichromate. The boiling temperature, however, breaks this up into silver chromate and chromic acid.

2. *Magnesium Carbonate.*

3. *Potassium Iodide.*—50 per cent solution. Place 50 gm. of potassium iodide in a 100 cc. volumetric flask. Add distilled water to 80 to 90 cc. and dissolve the iodide, then make up to 100 cc. Filter if necessary.

4. *Sulfuric Acid.*—Prepare a 1:10 dilution of the concentrated acid in distilled water.

5. *Standard Sodium Chloride Solution.*—Weigh accurately 500 mg. of sodium chloride of highest purity and place in a liter volumetric flask. Dissolve in chloride-free distilled water and make up to 1 liter with chloride-free water.

#### *Procedure.*

Place 5 cc. of blood filtrate, obtained by the Folin and Wu technique, in a thoroughly cleaned 15 cc. conical centrifuge tube. Add a little (5 to 10 mg.) magnesium carbonate to insure absence of free acid and stir into the liquid with a fine glass rod. Add an excess (20 to 25 mg.) of silver chromate and stir thoroughly with a fine glass rod for 2 or 3 minutes. The stirring should include considerable rubbing of the silver chromate particles against the sides and bottom of the tube, as otherwise they may become coated with silver chloride and the reaction be incomplete. If all the red silver chromate particles disappear, add more silver chromate. Wash off the stirring rod into the tube with a little distilled water and centrifugate 2 or 3 minutes at moderate speed. Decant through a small filter into a 25 cc. volumetric flask. Add about 5 cc. of distilled water to the tube, running the water slowly and carefully down the side of the tube so as to disturb the sediment but little. (If the sediment is stirred up very much, centrifugate again.) Decant through the filter. With care both decantations may be made very complete. Filtration is made necessary by the tendency of very small particles of the silver chromate to float on the surface of the liquid, even during centrifugation. A slight turbidity, due to unremoved silver chloride, remains, but this clears later, after the addition of po-

tassium iodide. Wash the filter with several small portions of distilled water, but keep the volume in the flask below 23 cc. Add to the solution in the flask 1 cc. of 50 per cent potassium iodide solution and 1 cc. of 1:10 dilute sulfuric acid, make up to 25 cc. with distilled water, and mix. Compare the solution in the colorimeter with the standard.

Prepare the standard simultaneously with the test by placing 5 cc. of the standard sodium chloride solution in a 15 cc. conical centrifuge tube and carrying through the procedure exactly as described for the test.

$$\frac{\text{Reading of standard in mm.}}{\text{Reading of unknown in mm.}} \times 500 = \text{mg NaCl per 100 cc. of blood.}$$

$$\text{Mg. NaCl} \times 0.606 = \text{mg. Cl.}$$

TABLE I.

Sample No	Colorimetric method	Titration.
1	488	491
2	494	510
3	484	481
4	478	482
5	493	502
6	513	527
7*	590	579
8*	564	567
9*	594	592
10*	567	576

\* Plasma.

The color developed by this method reaches a maximum in a few seconds and remains unchanged for many hours. One standard may be used for many determinations.

One may, if desired, use a standard chromate solution as suggested by Isaacs instead of developing the color each time from the standard sodium chloride solution. The writer prefers the use of the sodium chloride standard as it, in large measure, compensates for the slight solubility of silver chromate in water. A potassium chromate standard adjusted to the new method should contain 0.835 gm. of potassium chromate per liter of solution. Place 5 cc. of this solution in a 25 cc. volumetric flask, add water to about 20 cc., then 1 cc. of 50 per cent potassium iodide solution

and 1 cc. of 1:10 dilute sulfuric acid. Make up to 25 cc. with distilled water and mix. The color is approximately the same as that given by 5 cc. of the standard sodium chloride solution, containing 500 mg. per liter, that has been carried through the method given. Its exact value should be determined by comparison against the standard sodium chloride.

This method has been checked against the titration method of Gettler (1921) and of McLean and Van Slyke (1915) and the results of ten such tests are shown in Table I. The chlorides are computed as mg. of NaCl per 100 cc. of blood or plasma.

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## DIETARY REQUIREMENTS FOR REPRODUCTION.

### I. THE NUTRITIVE VALUE OF MILK PROTEINS FROM THE STANDPOINT OF REPRODUCTION.\*

By BARNETT SURE

*(From the Laboratory of Agricultural Chemistry, University of Arkansas, Fayetteville )*

(Received for publication, October 20, 1923 )

During 1919, while engaged in studies of supplementary relationship of lactalbumin to casein in milk, the author observed that on a ration containing 9.6 per cent milk proteins very good growth was obtained, but the females, while they became pregnant and gave birth to healthy young, failed to rear them. That ration (Lot 22) contained 7 per cent butter fat and an alcoholic extract of 15 gm. of ether-extracted wheat embryo as a source of water-soluble vitamin B; therefore, it was thought at that time that the vitamins in the ration were amply provided for. Steenbock's salt mixture 32 (the composition of which is given later in the paper) served as such an excellent source of the mineral elements in Ration 11 (see Chart I) for growth that a deficiency of the inorganic ions in Ration 22 certainly was not expected. It also became quite evident at that time that cystine is the determining growth-limiting factor in lactalbumin (1), Osborne and Mendel having previously demonstrated cystine to be a deficient amino-acid in casein at levels below 12 per cent of protein intake (2). Owing to the fact that we do not as yet know the amino-acid requirement of the mammalian organism for the physiological function of reproduction as contrasted with growth, it was anticipated that the further fortification of the milk proteins with cystine might result in some improvement in reproduction from the

\*Research paper No 2, Journal Series, University of Arkansas.

A preliminary report of this paper has been presented before the Biochemical Division of the American Chemical Society at Milwaukee, September 13, 1923

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standpoint of rearing of young. Later on, experiments were initiated for the purpose of improving the quality of milk proteins by additions of other amino-acids, such as lysine and proline, particularly during the breeding period, and if possible, during the lactation period. The results of these investigations are published in this paper.

While these experiments were in progress, Mattill and Conklin (3), Mattill (4), and Mattill and Stone (5) have reported their unsuccessful efforts with milk diets from the standpoint of reproduction. A careful scrutiny of the rations of these experimenters indicates that their diets had a suitable inorganic salt mixture and an abundance of the fat-soluble and water-soluble vitamins. At the 1921 annual meeting of the American Society of Biological Chemists, Mattill reported as follows:

"The failure of adolescent growth and of reproductive ability in female rats on whole milk powder was not corrected by diluting the milk powder with lard, starch, and salts in varying proportions. The animals still showed the characteristic slowing of growth rate at adolescence and were infertile. Males grew normally but their reproductive efficiency was questionable. Additions of protein-free milk, of cod liver oil, or of traces of K<sub>1</sub> after the animals had reached the age of 150 to 175 days did not result in fertility or renewed growth. Ordinary stock rat food did not do it. When animals that grew up on stock food were transferred to milk rations at about the age of adolescence they likewise failed to rear their young, or were sterile."

Owing to the fact that Mattill and his coworkers, in order to obtain success in reproduction with milk diets, have not attempted to improve the protein moiety, the author still hoped to obtain success along this line by amino-acid additions to his milk protein rations.

In the earlier experiments conducted at the University of Wisconsin butter fat was used as the source of fat-soluble A vitamin. In all the other experiments, initiated and completed at the University of Arkansas, cod liver oil, found by Zilva and coworkers (6) to be 200 to 300 times richer than average butter fat in fat-soluble A, was employed as a source of that vitamin. The water-soluble B vitamin concentration of these rations was increased from an alcoholic extract of 15 gm. of wheat embryo to 30 and 40 gm. per 100 gm. of ration. It was calculated that in rations, containing 50 per cent skimmed milk powder, about 3.5

per cent of mineral elements would be introduced; therefore, no salt additions were made in such diets. Rations containing 35 per cent skimmed milk powder, however, were fortified with 1 per cent of salt mixture 32. Since milk is deficient in iron, 0.1 per cent of ferric citrate was added to all the milk powder rations. After making, what seemed to the writer, ample provisions for vitamin and mineral requirements, all the rations containing an abundance of digestible carbohydrates in the form of dextrin, it was naturally expected that the reproductive mystery of milk diets from the standpoint of reproduction would be solved by improving the amino-acid content of the milk proteins.

The results of the experiments are given in the charts and protocols.

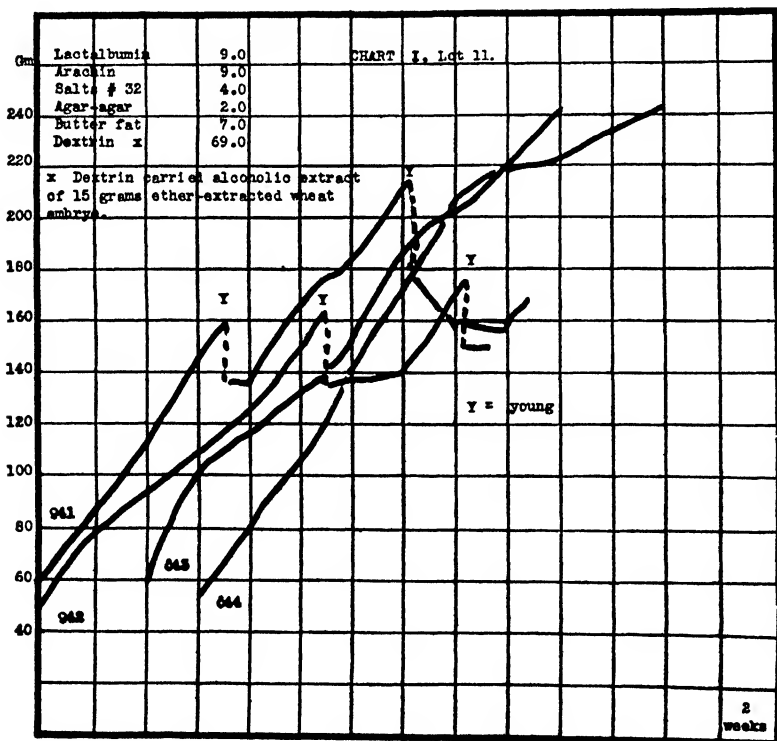


CHART I, LOT 11. Although lactalbumin (1) and arachin (7) have both proved to be deficient proteins for growth, arachin was found to be such an efficient supplement to lactalbumin for growth, that the experiment

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was continued with a view of securing, if possible, data on reproduction. Female 41 gave birth to eight healthy young, but was given only four to rear, so as not to overburden the mammary gland. All the young were disposed of in 10 days. 7 weeks later the same mother gave birth to another litter of six young and was allowed four to rear. After 2 weeks the young weighed 69 gm. They looked perfectly vigorous, had a smooth coat, and after 15 days opened their eyes. 7 days later this group of young weighed only 64 gm, having lost 5 gm within a week. On the 16th day one of the young died and the rest died within the next few days. Female 42 gave birth to a litter of seven young and successfully reared four for 2 weeks, after which period the young were completely devoured by the mother. The same female gave birth to another litter which was disposed of during the day of delivery.

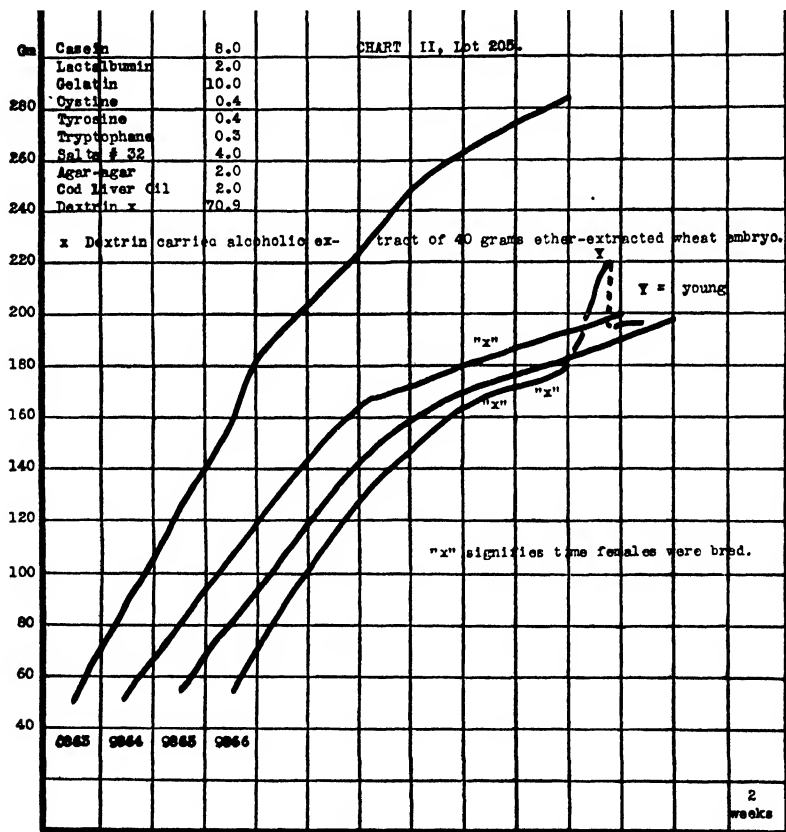


CHART II, Lot 205. This ration certainly had a preponderance of the water-soluble B vitamin, containing a hot alcoholic extract of 40 gm. of

ether-extracted wheat embryo. The proteins were fed at a 20 per cent level, containing 8 per cent casein, 2 per cent lactalbumin (the casein and lactalbumin being fed in the same proportion as they are found to occur in milk), and 10 per cent gelatin. The ration was further fortified with the amino-acids, cystine, tyrosine, and tryptophane. 2 per cent cod liver oil certainly should have been sufficient for all physiological functions, and salt mixture 32 has been shown to be of superior quality (see Chart I, Lot 11). Still, on such a diet only one female out of three became pregnant and gave birth to only two young which were disposed of by the mother in a few days

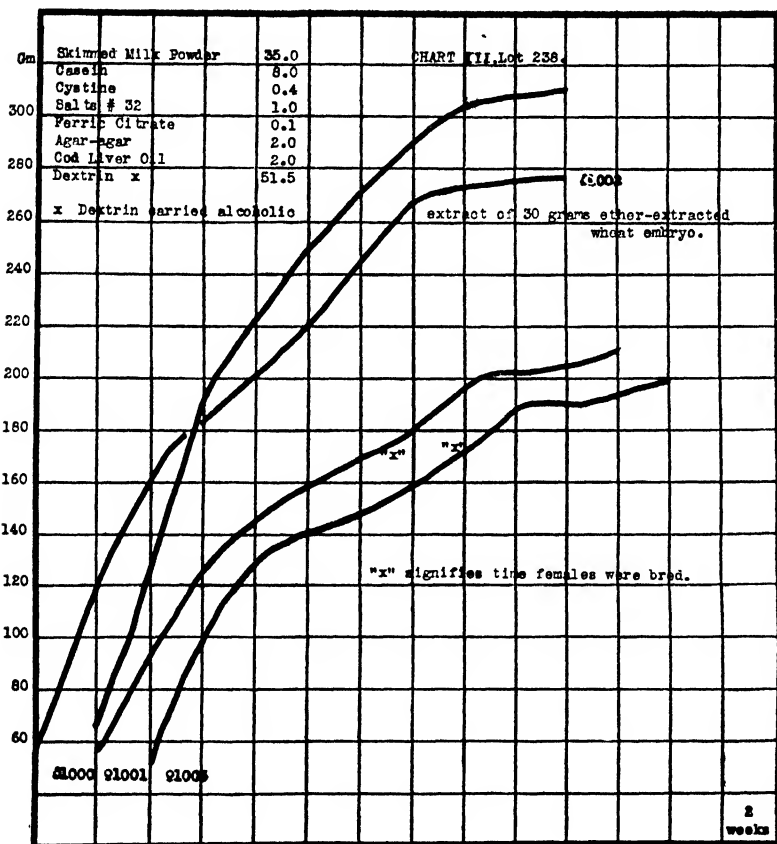


CHART III, LOT 238. This ration contained 35 per cent skimmed milk powder, having a protein content of 34 per cent, which would introduce 11.9 per cent milk protein. The ration was fortified with additional

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8 per cent casein and 0.4 per cent cystine. While excellent growth was obtained, the females did not even become pregnant.

Lot 233. This lot received the same ration as Lot 238 with the exception that lactalbumin replaced the casein. No fertility was secured on this diet.

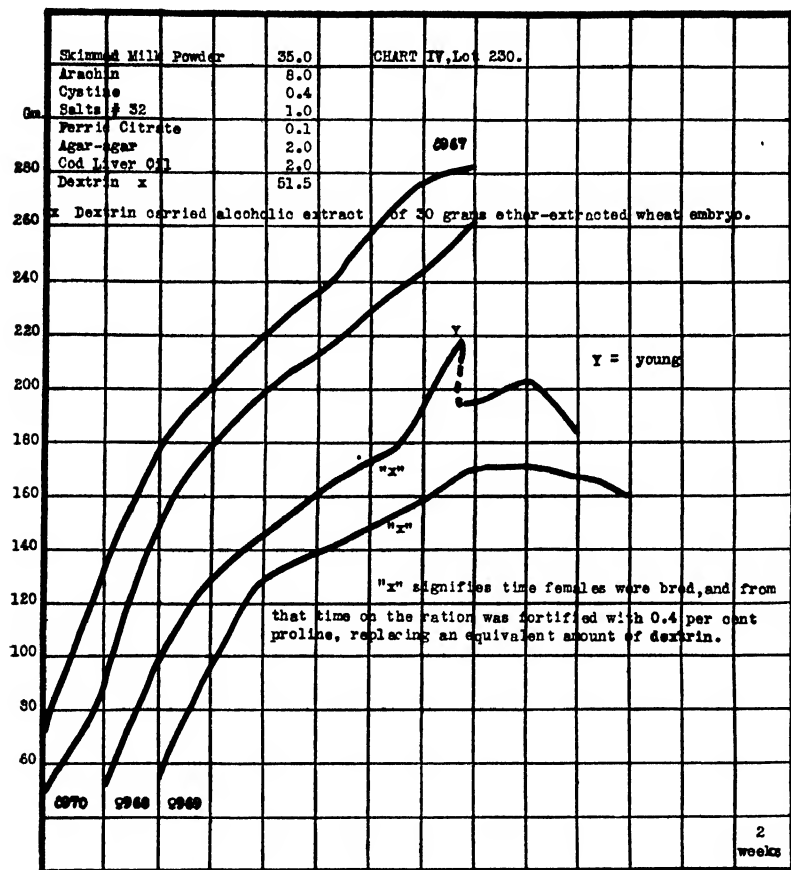


CHART IV, Lot 230. The skimmed milk powder in this ration was fortified with 8 per cent arachin (globulin from the peanut) and 0.4 per cent cystine. Since arachin is very low in proline, containing only 1.37 per cent, that amino-acid was added during the breeding period. Only one female became pregnant, giving birth to only one young, and, therefore, rearing was not tried.

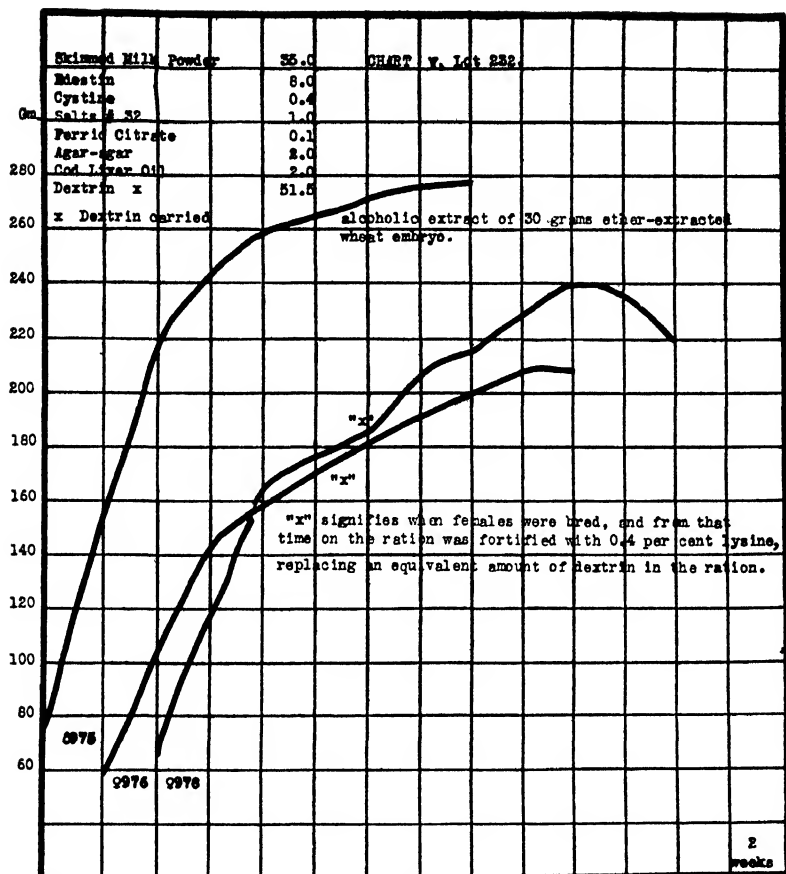


CHART V, LOT 232 This ration had the addition of 8 per cent edestin (globulin from hemp seed) and 0.4 per cent cystine to the milk proteins, and since edestin is deficient in lysine, that amino-acid was incorporated in the diet during the breeding period. Very good growth was secured, but the females were sterile.

LOT 254. This ration contained 22 per cent protein, 12 per cent of which was derived from milk, and 10 per cent from wheat, the wheat proteins being introduced in the form of wheat gluten. No fertility was secured.

LOT 253 This ration contained 32 per cent protein, 12 per cent of which was fed in the form of skimmed milk powder, and 20 per cent in the form of wheat gluten. The females did not become pregnant.



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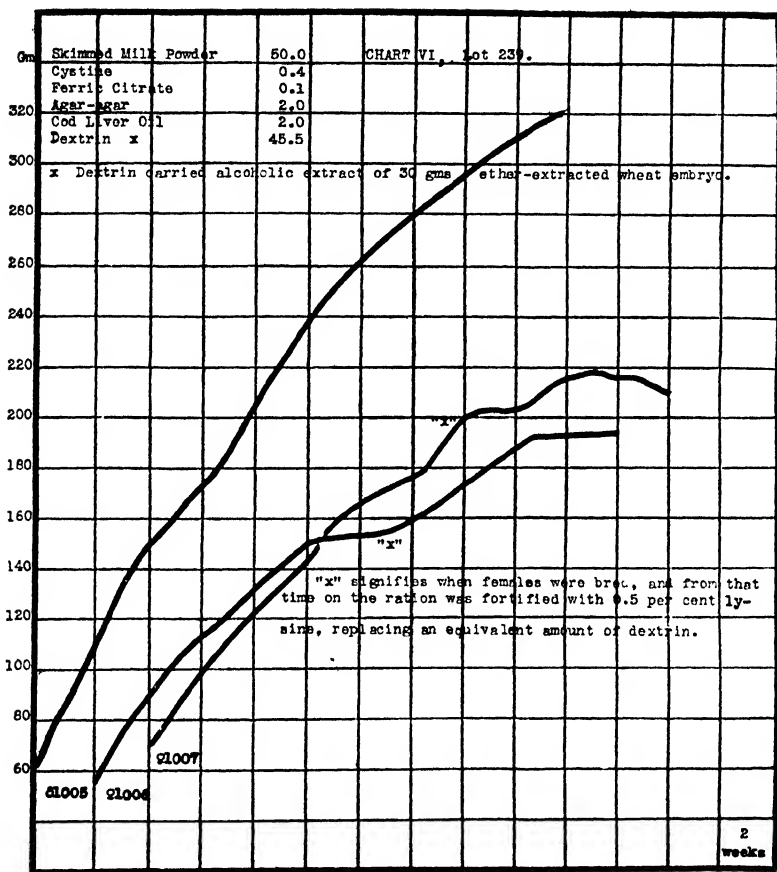


CHART VI, Lot 239. This chart shows that the addition of lysine to 17.5 per cent milk protein-cystine diet did not produce fertility.

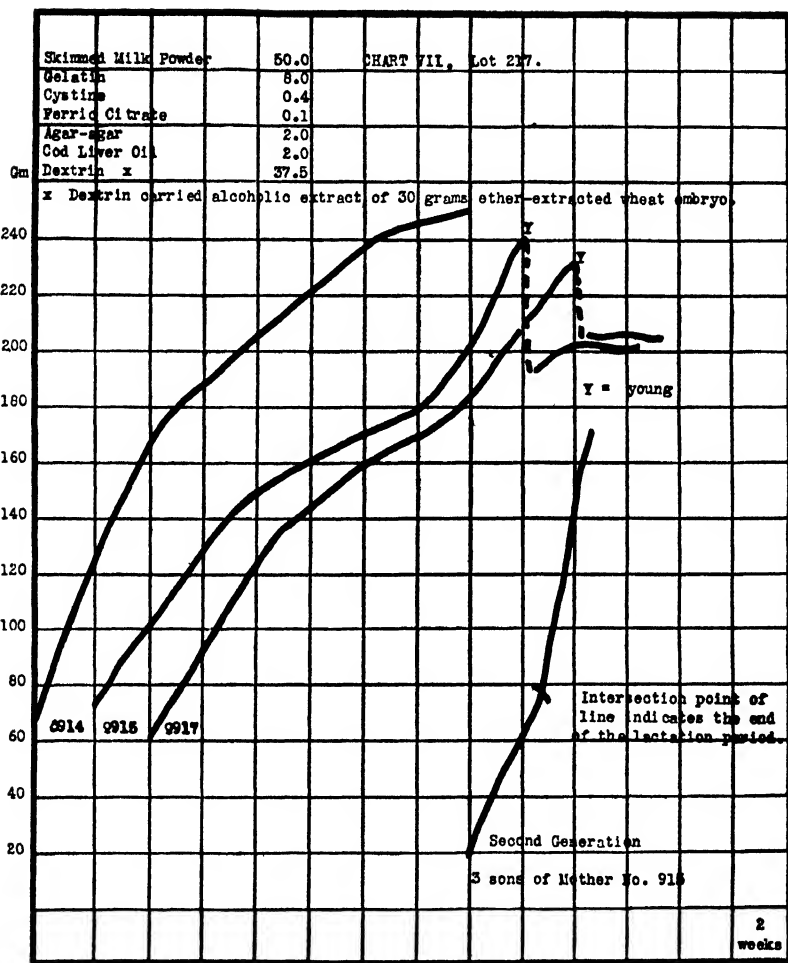


CHART VII, LOT 217. This is the only skimmed milk powder ration on which partial success in reproduction was obtained. The foreign protein introduced is gelatin. Cystine was added to the extent of 0.4 per cent of the ration. Rat 917 gave birth to a litter of young which was devoured by the mother. Female 915 gave birth to seven young and was given four to rear. On the day after delivery one of the young died which left three to rear. These young weighed 29 gm after the 1st week, 48 gm after the 2nd week, and 73 gm after the 3rd week.

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### DISCUSSION.

That milk proteins, while being very good for growth, are inadequate for reproduction, has been pointed out in a previous communication (8). This paper deals with a strenuous attempt made to induce fertility and success in rearing of young by improving the amino-acid content of milk proteins, both in quantity and quality, and at the same time by satisfying the vitamin content of the ration by feeding liberal amounts. The salt mixture employed was that introduced by Dr. Steenbock which he designated as salts 32, having the following composition.

	<i>gm</i>
NaCl	0 2022
MgSO <sub>4</sub> (anhydrous)	.. . 0 3117
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O	0 5265
K <sub>2</sub> HPO <sub>4</sub>	1 1158
Ca <sub>2</sub> H <sub>2</sub> (PO <sub>4</sub> ) <sub>2</sub> 4H <sub>2</sub> O	1 1165
Calcium lactate .	0 2896
Ferric citrate	0 1385

Such a proportion of the inorganic elements, fed to the extent of 4 per cent in the ration, proved itself to be very satisfactory in our synthetic diets for growth. Whether additional inorganic elements or different proportions of the same inorganic ions are required for the physiological function of reproduction as contrasted with growth is still open for investigation; but, while such a fact may be possible, it is hardly probable on the basis of work of other investigators which will be discussed in the following paper.

In addition to the mineral elements derived from the above salt mixture all our animals receive iodine weekly in their distilled water. A saturated solution of iodine dissolved in a 2 per cent solution of potassium iodide is kept as a stock solution. 20 cc. of this solution are added to about 18 quarts of distilled water, the watering tubes being filled once a week from that solution. The water has a faint color of iodine.<sup>1</sup>

The milk proteins fed at a 11.9 per cent level, fortified with 8 per cent casein, arachin, or edestin, in the presence of 0.4 per

<sup>1</sup> Such a method of furnishing iodine to our animals has been secured from a personal communication kindly furnished by Dr. McCollum

cent cystine, did not improve the matter of fertility and success in rearing of young. In certain cases lysine or proline were added during the breeding period, and still the females did not become pregnant.

When 17.5 per cent milk proteins were fortified with 8 per cent gelatin, in the presence of 0.4 per cent cystine, partial success in

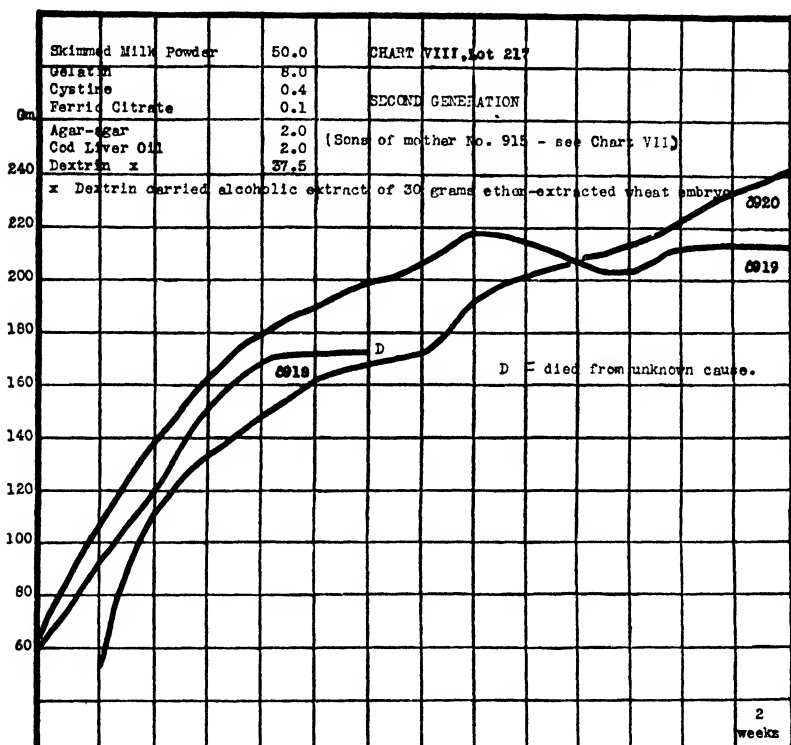


CHART VIII.

reproduction was obtained, and for a while it was thought that sterility on milk diets could possibly be explained by the deficiency of amino-acids. One of the mothers on this ration (Chart VIII, Lot 217) reared three young at a rate which may be considered as a little better than half normal. These three young were able to help themselves at the termination of the lactation period, and after 5 weeks were separated from their mother and

made excellent growth. One of the young, however, died from an unknown cause at the age of 4 months. It was unfortunate that these animals were males, so that a third generation could not be hoped for. Another group of three young females, taken at weaning time, was brought up to sexual maturity on the same ration, and allowed to breed with the two remaining males. No fertility was secured. Since no anatomical analysis was made of the animals, it is not apparent whether the sterility is to be attributed to the males or the females. The fact, however, is that the females did not become pregnant after being allowed to breed for over 2 months.

Even increasing the total quantity of protein to 32 per cent, 12 per cent being derived from skimmed milk powder and 20 per cent from wheat gluten, in the presence of a liberal supply of the vitamins and a suitable salt mixture, did not result in any success in reproduction.

#### SUMMARY.

From the experimental data presented in this paper it is concluded that lack of fertility or significant success in rearing of young on milk diets must be attributed to a dietary factor other than protein, the fat-soluble A vitamin, the antirachitic vitamin, or the water-soluble B vitamin. Whether failure in reproduction on synthetic diets is to be ascribed to a deficiency in the mineral element complex will be discussed in the following paper.

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## DIETARY REQUIREMENTS FOR REPRODUCTION.

### II. THE EXISTENCE OF A SPECIFIC VITAMIN FOR REPRODUCTION.\*

BY BARNETT SURE.

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Fayetteville )

(Received for publication, October 20, 1923 )

During 1922, at the time the experiments reported in this paper were nearing completion, Evans and Bishop announced in Science "The existence of a hitherto unrecognized dietary factor essential for reproduction." They state (1):

"The fact has been abundantly demonstrated that rats may be reared on a dietary regime consisting of 'purified' protein, fat and carbohydrate to which an appropriate salt mixture and adequate doses of the growth vitamins Fat Soluble A and Water Soluble B have been added. We have employed a ration of casein (18), cornstarch (54) and lard (15) to which butterfat (9) and salts (4) are added, the animals receiving separately and daily 4 gram each of dried whole yeast

"Such animals are sterile. They are chiefly so in the first generation and wholly so in the next succeeding one

"Natural foodstuffs contain a substance, X, which prevents such a sterility or which cures the disorder occasioned by the purified dietary regime. We have thus been able to witness a comparatively sudden restoration of fertility to animals of proven sterility, and whose controls continued sterile, by the administration of fresh green leaves of lettuce. Even the dried leaves of alfalfa appear to possess a similar potency "

Several papers by the same authors (2, 3) have since appeared "On the relations between fertility and nutrition," showing their failures with reproduction on synthetic rations and the restoration of fertility accompanied by successful rearing of succeeding genera-

\*Research paper No. 3, Journal Series, University of Arkansas.

A preliminary report of this paper has been presented before the Biochemical Division of the American Chemical Society at Milwaukee, September 13, 1923, under the title "Suggestive evidence for the existence of a specific vitamin for reproduction." The accumulation of more experimental data justified the omission of the words "suggestive evidence."

## 694 Dietary Requirements for Reproduction. II

tions by the introduction of at least one natural foodstuff to their diets of purified food substances. They find this hitherto unrecognized dietary factor essential for reproduction to be present not only in green lettuce and dried alfalfa leaves, but also in wheat, oats, meat, and, to a lesser extent, in milk fat.

Evans and Bishop's work<sup>1</sup> is in harmony with the author's findings that lactalbumin, replacing casein in the ration, does not induce fertility; neither is fertility restored by increasing the casein content to 50 per cent.

Since there is no experimental evidence available, in terms of specific dietary units, in connection with the requirement of water-soluble B vitamin for the physiological function of reproduction as contrasted with growth, it may be inferred that the failure to secure success with reproduction on purified food substances may be due to an insufficient intake of that vitamin, rather than to the deficiency of an additional unidentified dietary factor. Nelson, Heller, and Fulmer state in a recent publication (5) that even upon 8 per cent air-dried yeast, which they found to be four times the amount necessary for normal growth to maturity, the rearing of young is not entirely successful, and that failure in reproduction cannot be ascribed solely to the level of vitamin B. These investigators, however, report success with yeast from the standpoint of reproduction when the level is increased to 30 to 45 per cent. Three generations have been obtained on these high yeast levels, although the young of the second and third generations have not grown normally. They ascribe the failure of Evans and Bishop to secure fertility, employing 25 per cent yeast, to the high fat content of their diets. Such an explanation is hardly reasonable since Evans and Bishop first begin to secure success in reproduction with milk fat when they increase it to 24 per cent of the ration. Since yeast has been shown to be abundant in the water-soluble B vitamin, why is it not possible that, when it is fed at such a high level as 30 per cent, it may introduce some of the as yet unrecognized syndrome that is essential for reproduction?

Is it possible that the failure in reproduction on synthetic diets may be ascribed to neglect to make proper provisions for the mineral element complex? That a sufficient amount of the inorganic elements is added when 3.7 per cent of a salt mixture is

<sup>1</sup> Evans and Bishop (4), p. 273.

employed in a ration is evident from the work of Nelson, Heller, and Fulmer. On the contrary, increasing their salt mixture 185 to 5 per cent, they find their animals without exception to be sterile, although they grow at the normal rate to maturity. Purified milk fat is certainly free from any significant amount of mineral elements, yet, keeping the amount of the salt mixture in their basal ration constant, but increasing the milk fat to 24 per cent, sterile females become pregnant and manifest a certain degree of success in rearing their young.

Evans and Bishop's findings<sup>2</sup> agree with those of Mattill and coworkers and with those of the author; that a preponderance of cod liver oil, in the presence of adequate concentrations of all the other recognized dietary essentials, does not produce fertility and success in rearing young.

The experimental data which convinced the author that there must exist an as yet unrecognized dietary factor essential for reproduction are summarized in the charts

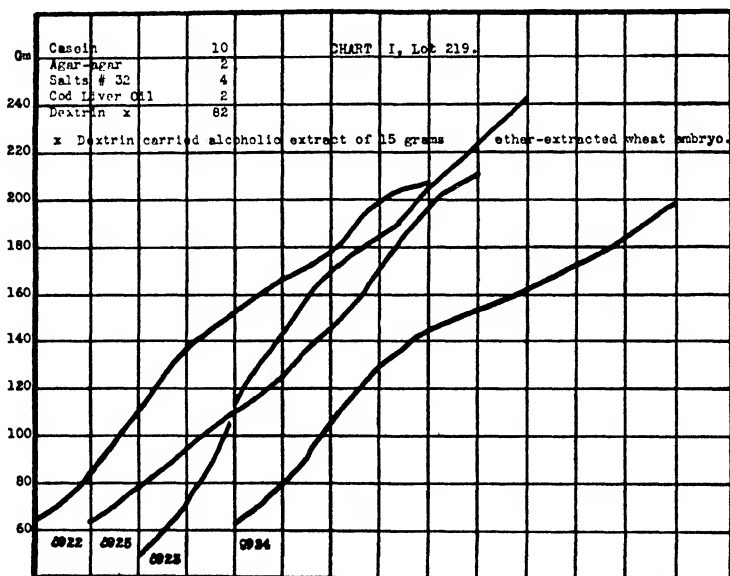


CHART I, Lot 219. This chart clearly shows that, employing an alcoholic extract of 15 gm. of ether-extracted wheat embryo as a source of water-soluble B vitamin, very good growth is obtained.

<sup>2</sup> Evans and Bishop (3), pp. 202-231, (4), pp. 233-273.



## 696 Dietary Requirements for Reproduction. II

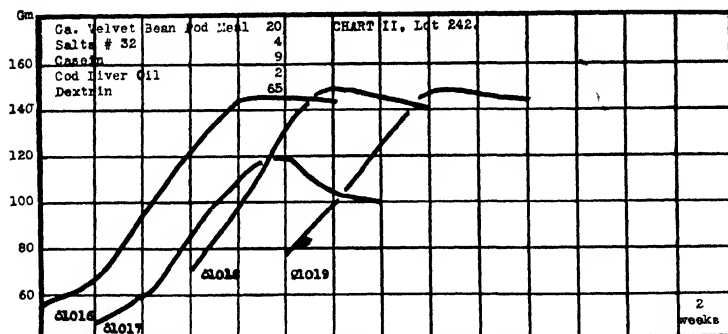


CHART II, Lot 242 20 per cent of the Georgia velvet bean pod meal as a source of water-soluble B allows fairly good growth to take place during the first 6 to 8 weeks of experimentation, but the growth is followed by rapid decline in body weight

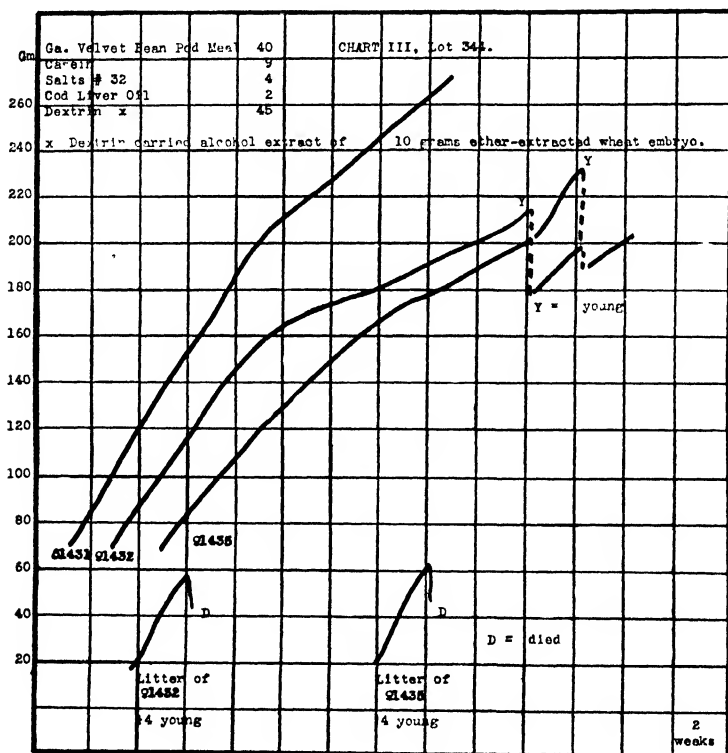
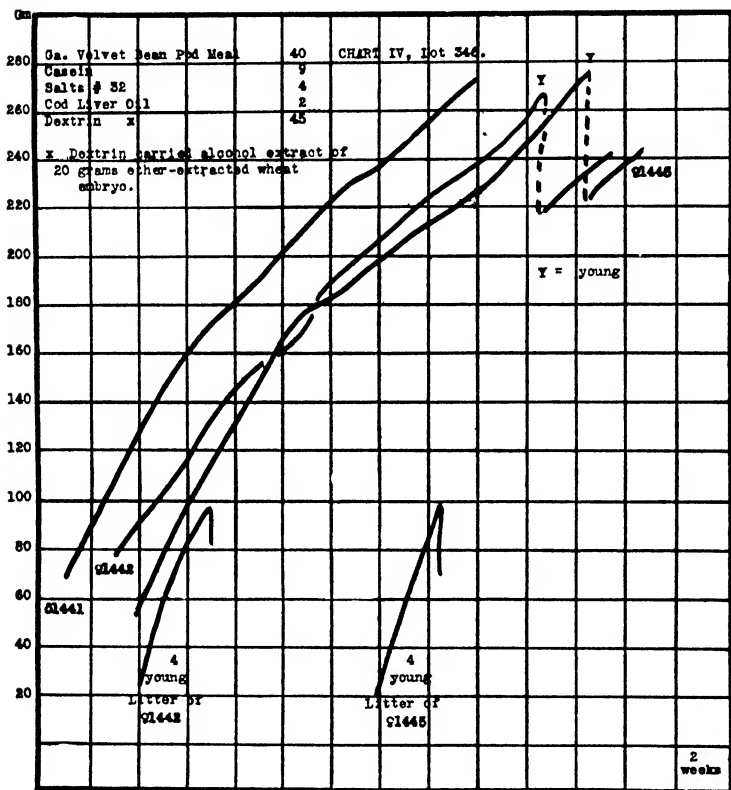


CHART III, Lot 344 This chart shows that fertility is secured when 40 per cent of the ration is composed of velvet bean pod meal, in which diet is

incorporated an alcoholic extract of 10 gm. of wheat embryo. Female 1432 had six young, and was allowed four, weighing 20 gm., to rear. The young reached a maximum weight of 56 gm, but on the 15th day there was a rapid decline of body weight followed by death. Female 1435 had five young, and was given four, weighing 20 gm, to rear. The young reached a maximum weight of 63 gm., but died on the 15th day



**CHART IV, LOT 346.** This chart clearly indicates the increase of success in rearing of young of two females, due to the increase of the water-soluble B vitamin from an alcoholic extract of 10 to 20 gm. of wheat embryo. Female 1442 had eleven young and reared four for 18 days to a maximum weight of 94 gm. On the 19th day the young began to lose weight rapidly, and rearing was abandoned. Female 1443 had five young, and four, weighing 24 gm at birth, were reared to a maximum weight of 93 gm in 18 days, after which period rapid decline in weight of young necessitated the discontinuance of further rearing

## 698 Dietary Requirements for Reproduction. II

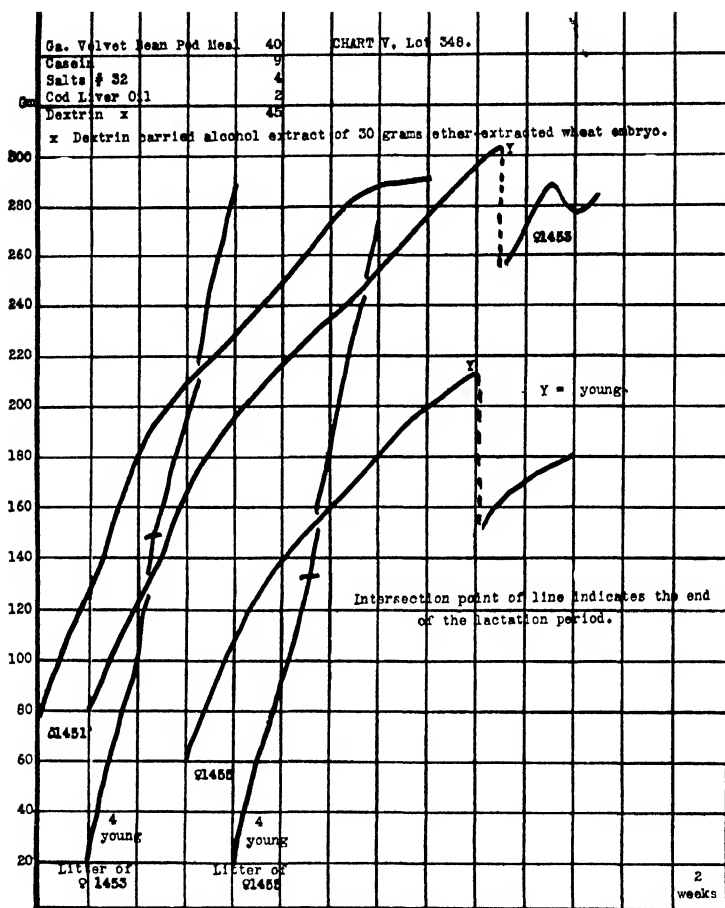


CHART V, Lot 348 Increasing the concentration of the water-soluble B vitamin to an alcoholic extract of 30 gm of ether-extracted wheat embryo resulted in a further improvement in success of rearing of young Female 1453 had ten young weighing 50 gm, and was given four, weighing 20 gm, to rear. At the end of 23 days, which marks the termination of the lactation period, the four young collectively weighed 150 gm, and at the end of 5 weeks, weighed 289 gm. Female 1455 had eleven young, weighing 50 gm, and was allowed four, weighing 19 gm, to rear. At the end of the lactation period the group reached a weight of 128 gm., and at the end of 5 weeks weighed 286 gm.

Charts III, IV, and V show the rôle water-soluble B vitamin plays in the matter of rearing of young, but also indicate that the fertility which may occur in the first place must be attributed to something contained in the velvet bean pod meal which is absent in rations composed of purified food substances. This matter will be elaborated on under the heading "Discussion."

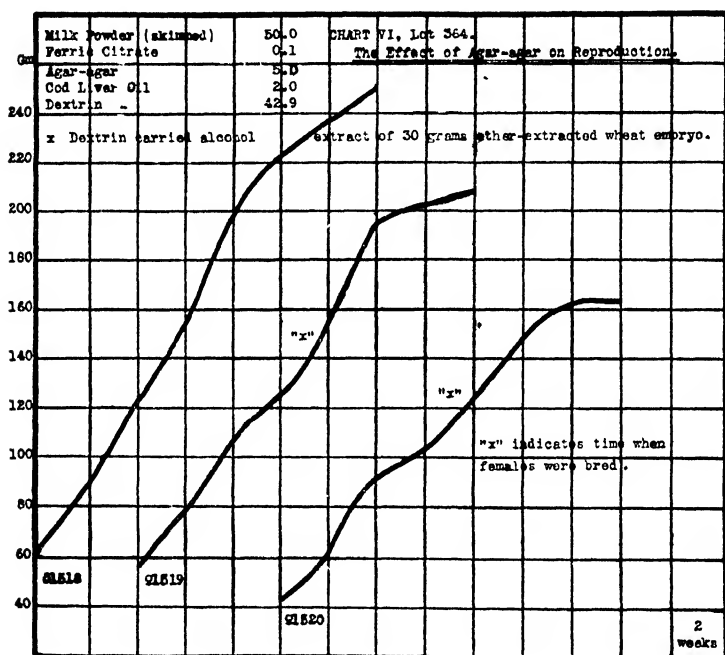


CHART VI, Lot 364 The presence of 5 per cent of agar-agar in a milk ration, fortified with an abundance of fat-soluble A and water-soluble B vitamins, does not induce fertility



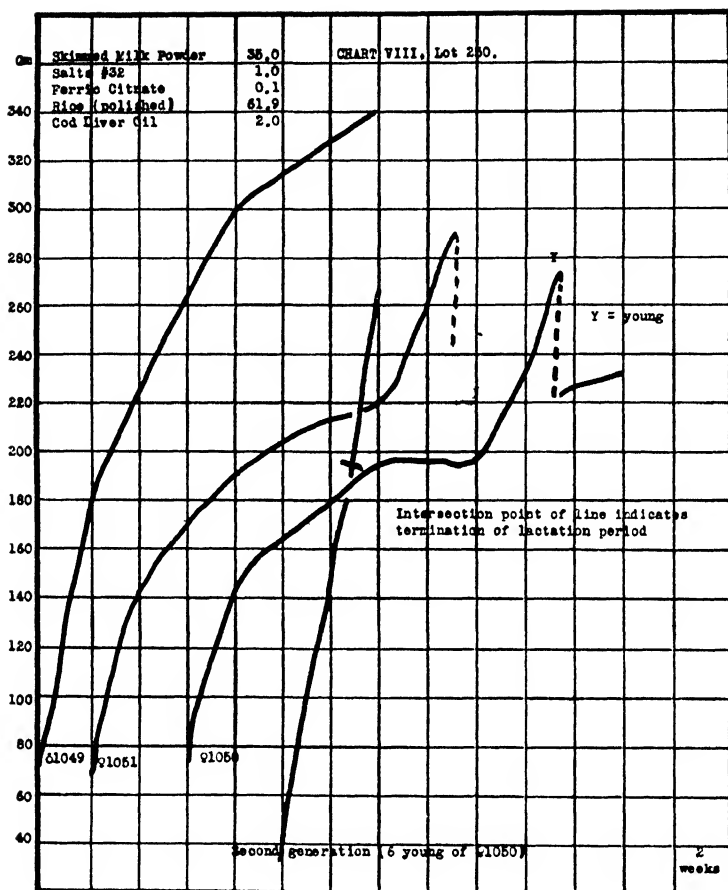


CHART VIII, Lot 250 Since polished rice is reported to have only traces of water-soluble B vitamin, the 35 per cent of skimmed milk powder furnished practically all of that complex in this ration. Female 1051 gave birth to three young which were disposed of on the 3rd day. Female 1050 gave birth to six young which were successfully reared, and on the 15th day weighed 152 gm. On the 21st day one of the young died, the five remaining young then weighing 192 gm. On the 23rd day another of the young died, leaving four, which collectively weighed 192 gm on the 24th day. On the 31st day after birth the remaining four young collectively weighed 263 gm. The remarkable result in this experiment is that on such a low concentration of water-soluble B fertility was secured with two females and successful rearing of four young at a normal rate.

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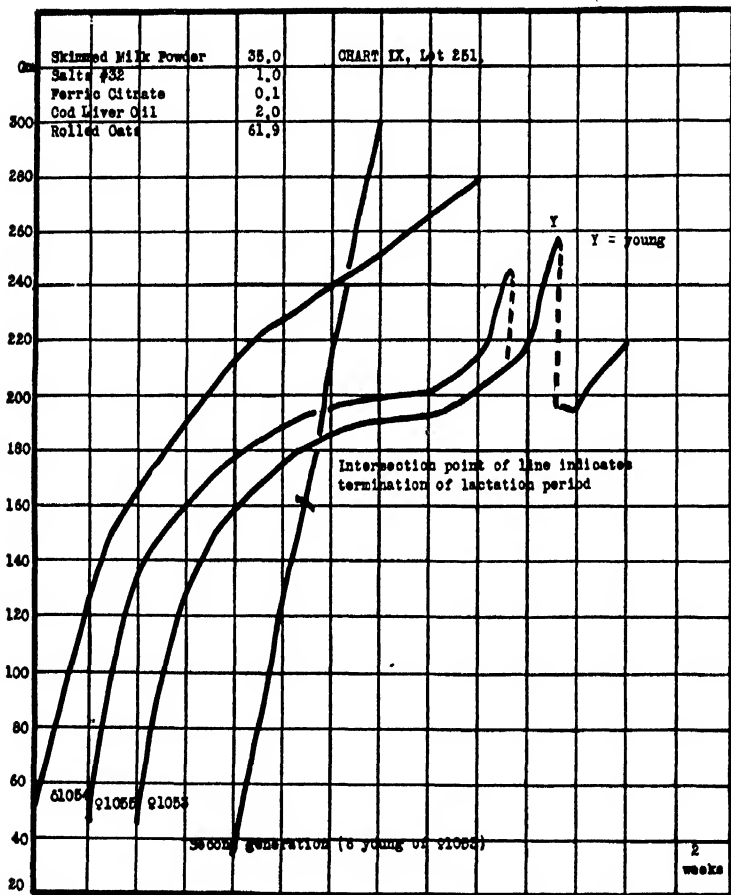


CHART IX, Lot 251. The natural foodstuff incorporated in this ration is rolled oats. Fertility with two females was secured and considerable success was obtained in rearing of young with one. Female 1055 gave birth to six young which were disposed of on the 2nd day. Female 1053 gave birth to six young, weighing 30 gm. At the end of the lactation period they weighed 160 gm.; on the 33rd day, 216 gm, and on the 41st day, 300 gm.

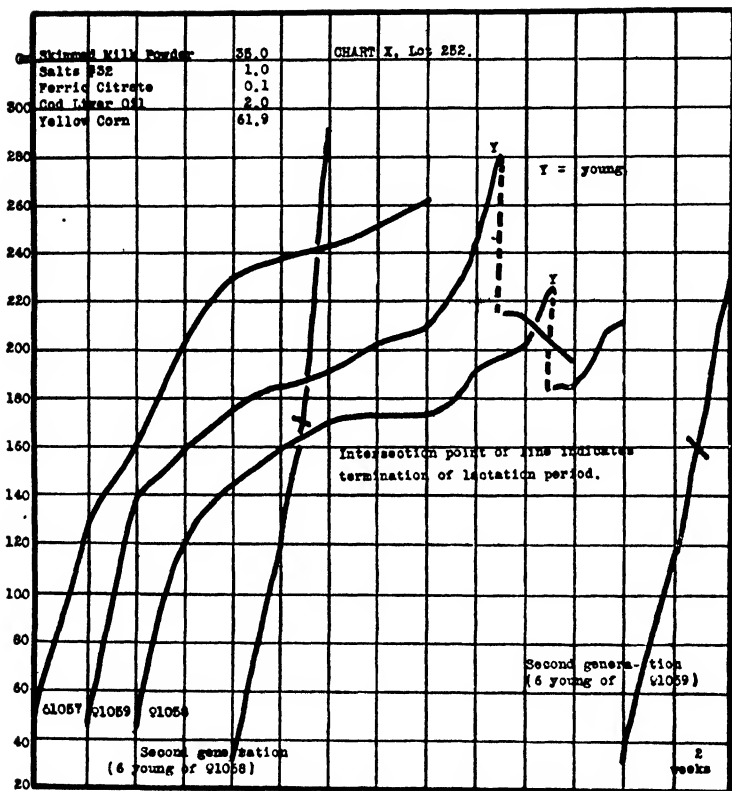


CHART X, LOT 252. Yellow corn is the seed introduced in this diet. Female 1058 gave birth to six young, weighing 30 gm, which, at the end of the lactation period, weighed 172 gm, and at the end of 31 days, 290 gm. Female 1059 gave birth to six young, weighing 33 gm, which, at the termination of the lactation period, weighed 160 gm, and at the end of 31 days, 232 gm.



## 704 Dietary Requirements for Reproduction. II

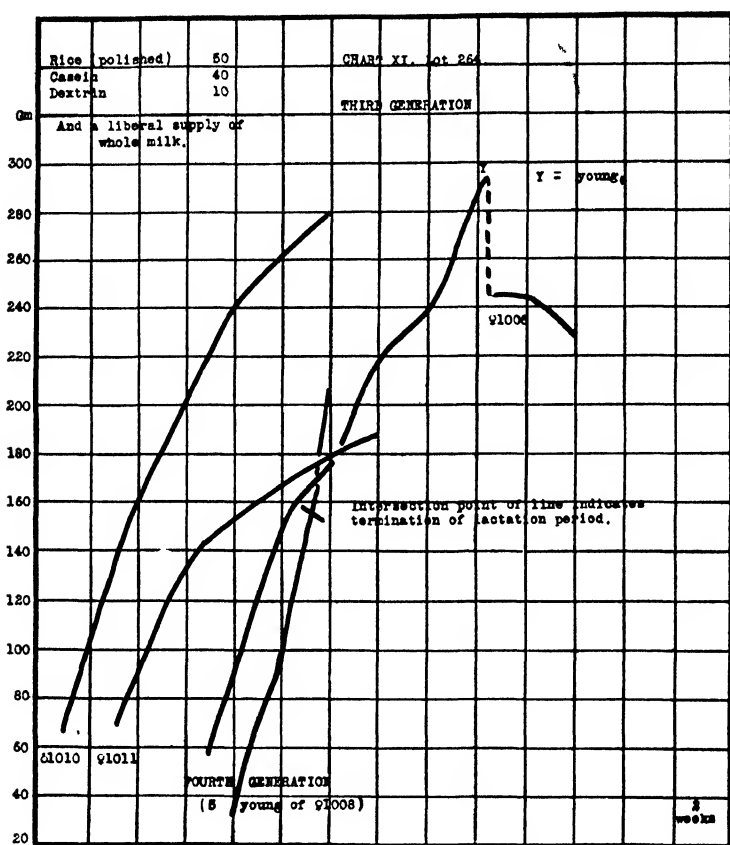


CHART XI, Lot 264 Although it has been shown that fortifying milk diets with protein, in the presence of liberal amounts of digestible carbohydrates, produces no success in reproduction, the addition of polished rice to such a dietary regime resulted in four successful generations

### DISCUSSION.

That the seed of the Georgia velvet bean is very deficient in water-soluble B vitamin has been demonstrated by Sure and Read (6). Unpublished work of J. W. Read on the biological analysis of Georgia velvet bean pod meal shows that 40 per cent of the pod meal as a source of water-soluble B allows as much growth to take place as that secured by the addition of an alcoholic extract of

15 gm. of wheat embryo. 20 per cent, however, of velvet bean pod meal as a source of that vitamin permits some growth during the first 6 to 10 weeks, but this is followed by rapid decline of body weight. A ration containing 40 per cent velvet bean pod meal and an alcoholic solution of 10 gm. of wheat embryo would be equivalent to a diet containing an alcoholic extract of 25 gm. of wheat embryo. On such a ration fertility was always secured, yet no success of rearing young resulted on a ration containing alcoholic extracts of 40 gm. of wheat embryo in the presence of such an excellent concentration of amino-acids as that furnished by the proteins, casein, lactalbumin, and gelatin, and the amino-acids, cystine, tyrosine, and tryptophane. (See Chart II of the preceding paper.) The inference made from such experiments is that the fertility and partial success of rearing of young on the velvet bean pod meal rations must be attributed to a dietary factor other than water-soluble B. Since the mineral elements, fat-soluble A, and the antirachitic factor were amply provided for in those rations (the rat being able to synthesize the water-soluble C vitamin (7)) the success of reproduction must be ascribed to a new unidentified factor which influences reproduction.

Lot 250, Chart VIII, discloses an interesting fact. In this ration no addition of water-soluble B vitamin was made, and polished rice, which is reported as being practically absent in that complex was the natural foodstuff introduced; therefore, the only source of that vitamin is the 35 per cent of skimmed milk powder. That 24 per cent of skimmed milk powder allows considerable growth to take place has been shown by McCollum and Davis in their paper, "The influence of the plane of protein intake on growth" (8), so it is not at all surprising that the 35 per cent of skimmed milk powder in this ration furnished considerable of the water-soluble B complex. The addition of an alcoholic extract of 30 gm. of wheat embryo, however, to a 50 per cent skimmed milk powder ration, in the presence of an abundance of all the other dietary factors, produced no fertility (see Chart VI, Lot 239, preceding paper). Therefore, the success with fertility and partial success in rearing of young on this ration must be ascribed to some new factor in polished rice that is essential for reproduction.

In the preceding paper experimental evidence has been presented showing the inability to secure fertility by improving the protein

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moiety of the milk diets both as to quantity and quality of amino-acids; yet, on a ration containing polished rice, 50, casein (commercial), 40, dextrin, 10, and a liberal supply of whole milk, four successful generations were secured, although the only foreign material added was the polished rice. The fourth generation is growing normally just as the former three generations did. I repeat and emphasize then that polished rice must contain some as yet unrecognized dietary factor that is essential for reproduction.

As shown in Charts IX and X, rolled oats and yellow corn play a similar rôle to polished rice in reproduction.

### *Effect of Agar-Agar on Reproduction.*

Recently Mitchell (9) reported that she secured success with reproduction by incorporating 5 per cent of purified agar-agar in the ration, replacing an equivalent amount of starch. The data indicated in Chart VI do not bear out her contention. The ration contained protein of excellent quality, a suitable salt mixture derived from milk, iron citrate to make up for the deficiency of iron in milk, a liberal supply of fat-soluble A, the antirachitic factor, and water-soluble B. 5 per cent of agar-agar was introduced in the diet, yet no fertility resulted.

### *Effect of Green Lettuce on Reproduction.*

Green lettuce, as claimed by Evans and Bishop, produces a pronounced effect on reproduction. It is unfortunate that more animals were not employed in this experiment. One female became pregnant but died during parturition, but the other reared four young, during the lactation period, at a normal rate.

While it is true that an insufficient number of females were employed in the experiments which had the addition of a single natural foodstuff, the data presented clearly show that fertility always resulted and that in each ration at least one mother manifested a significant degree of success in rearing her young, which seldom happened in any of the experiments in which synthetic diets of purified food substances were employed.

The data of this communication also bring out incidentally the rôle played by water-soluble B vitamin in reproduction. Increasing the level of the concentration of that vitamin from an

alcoholic extract of 10 to 30 gm. of ether-extracted wheat embryo, litters of four young of the mothers on those rations have been reared from a maximum weight of 60 to 150 gm. during the lactation period. On the highest level of water-soluble B intake the young were able to help themselves to food 3 weeks after birth and grew up to be large rats weighing over 70 gm. each at the age of 6 weeks.

*Stability of the Reproductive Dietary Complex.*

The Georgia velvet bean pod meal, with which significant success in reproduction<sup>3</sup> was secured on the higher planes of water-soluble B vitamin intake, was autoclaved for 1½ hours at 15 to 18 pounds pressure, because it was previously found that the velvet bean seed is toxic to rats when fed in the raw condition (6). It would, therefore, seem that this new dietary complex which plays such a significant rôle in reproduction is relatively thermostable.

*Nomenclature of the Reproductive Factor.*

It is now generally conceded that fat-soluble A signifies the antixerophthalmic vitamin, water-soluble B represents the anti-beri-beri and growth-promoting complex, and that water-soluble C indicates the antiscorbutic vitamin.

<sup>3</sup> In this connection the author would like to correct an erroneous conclusion drawn with respect to the rôle of cystine in reproduction in a previous publication (10). The data presented were of a preliminary character and a number of experiments were conducted to secure more evidence on this point. The technique then adopted was to add cystine during the second lactation period of the females, and note the degree of success of rearing of young, during that period when cystine was incorporated in the diets, as compared with that secured during the first lactation period when cystine was absent in the rations, and it seemed at that time that a small but definite improvement in rearing of young was apparent, which was attributed to the addition of cystine. Further attempts along that line do not bear out such results, and attention is called to the danger in employing such technique. A female rat may be more vigorous when it gets a little older and manifest some improvement in rearing its second litter without any dietary changes and no significance should be assigned to small differences in the degree of success of rearing young. The only safe method is to use controls, make specific additions to other experiments, and note the effect of such changes on fertility and rearing of young during the first lactation period, or several lactation periods if possible.

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In 1921, Funk and Dubin (11) reported the separation from vitamin B of a substance which stimulates the growth of yeast and streptococcus, which they provisionally call "D<sub>1</sub>." At the present time there is considerable controversy as to whether there actually exists a substance necessary for the growth of yeast. The work of Nelson, Fulmer, and associates seems to indicate that it is possible to grow yeast on a medium wholly synthetic in origin (12). MacDonald (13) maintains that the growth-stimulating substance for yeast which Funk and Dubin have provisionally named "D" cannot logically be placed in the classification of the vitamins, and is not an indispensable nutrient principle for yeast, since it is synthesized during the slow proliferation of yeast cells in a medium of purified nutrients.

In 1922 McCollum, Simmonds, Becker, and Shipley (14) produced evidence showing that oxidation destroys the fat-soluble A vitamin in cod liver oil without destroying another substance which plays an important rôle in bone growth. Coconut oil was shown to be lacking in fat-soluble A, since it will neither prevent nor cure xerophthalmia. This oil, on the other hand, contains a substance which stimulates the deposition of calcium salts in rickets in a manner similar to cod liver oil.

Since the experimental evidence of McCollum and coworkers on the separation of the antirachitic from the antixerophthalmic vitamin is clear and convincing, it is proposed that the term "D" be employed to represent the antirachitic factor, and since the term "vitamins" has hitherto been used to designate substances the chemical nature of which has not yet been disclosed, but which have specific physiological functions, the author feels that he is not premature in applying the term vitamin to this dietary complex essential for reproduction. Therefore, if the evidence presented by the author and that published by Evans and Bishop on the new dietary complex essential for reproduction is accepted as fully convincing, it is suggested that this factor be termed vitamin "E,"<sup>4</sup> instead of substance "X," as proposed by Evans and Bishop.

<sup>4</sup> It is, of course, not impossible that this dietary complex is a mineral, the physiological rôle of which has not yet been investigated.

## SUMMARY.

1. From experiments initiated  $4\frac{1}{2}$  years ago and recently completed, the results of which are embodied in this and the preceding paper, I conclude that, in addition to the antixerophthalmic, antirachitic, antiberi-beri, and antiscorbutic vitamins,<sup>5</sup> there exists another hitherto unrecognized vitamin, that is essential for reproduction, which becomes evident only in breeding experiments where rations composed of purified food substances are employed.

2. This reproductive vitamin has been found to occur in Georgia velvet bean pod meal, polished rice, yellow corn, and rolled oats.

3. If the term "D" be accepted for the designation of the antirachitic factor, it is proposed that the term "E" be adopted to represent this new dietary factor that influences reproduction.

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<sup>5</sup> In autoclaving the velvet bean pod meal for  $1\frac{1}{2}$  hours at 15 to 18 lbs. pressure the antiscorbutic vitamin certainly must have been destroyed, and still, with such treated pod meal, fertility and a significant success in rearing of young were possible. From such results it would seem that failure in reproduction should not be ascribed to water-soluble C, or the antiscorbutic vitamin.



## THE EXCRETION OF AMMONIA AND NITROGEN.

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In a previous article (Hubbard and Munford, 1922), a series of determinations of the excretion of acid and ammonia in a number of normal subjects and of subjects suffering from achlorhydia was discussed. It was concluded that the concentration of ammonia in the urine approximately paralleled the reaction, and that, if the reaction was constant, the excretion of ammonia varied with the volume. Since the article was published Marshall and Crane (1922) have reported experiments upon the differential excretion of ammonia in operated dogs when one kidney was excreting a large volume of fluid and the other a smaller volume. Under the conditions of their experiments the concentration and rate of ammonia excretion did not vary with the reaction and volume of the urine excreted from the different kidneys. Since the results of Hubbard and Munford were based on a statistical study, while those of Marshall and Crane were founded on controlled experimental data, it has seemed best to analyze a more extensive collection of data than that previously discussed to see whether the results on normal human subjects were confirmed.

One series of results of this kind has already been reported (Hubbard and Allen, 1923). In this series the concentration of ammonia varied with the reaction, and an agreement between the rate of ammonia excretion and volume was demonstrated when the reaction was constant, but this last agreement was not so close as it was in the experiments studied earlier. In the results discussed below further data on these points are presented, and an attempt is made to determine whether the nitrogen excretion, which in general agrees roughly with the volume, accounted for the agreement noted between volume and rate of ammonia excre-



tion. The ratio between ammonia and nitrogen, which Hasselbalch (1916) found to agree more closely with the reaction than did the concentration of ammonia, has also been studied.

The subject of the experiment was a normal man (the author), who did not take active exercise during the periods. Specimens of urine were collected at hourly intervals by the method of Marshall (1922). In most instances these specimens were collected only through the morning, but one series which was collected throughout the day has been included. Protocols of some of the results have been published (Hubbard, 1922). Results on two specimens collected after the injection of adrenalin chloride have been included (the results found on analysis of these specimens were strictly comparable with the others), but otherwise the different experiments differed from each other only by differences in the food taken. The results are based upon two series of experiments, one carried out in July, 1922, and one in June, 1923. The amounts of ammonia excreted were rather different in the two series, and tables similar to those presented below were prepared from each series separately. These tables showed practically the same things as did the ones discussed, except that the positive findings were a little more clearly defined, but, as there was no good reason which could be assigned for not considering all the results as a whole, they have been so considered in this paper. Total nitrogen was determined by the method of Folin and Denis (1916) slightly modified to permit the use of the oxidizing reagent of Folin and Wu (1919). Other determinations were made as has been previously described (Hubbard and Munford, 1922). All analyses were made within a few hours of the time when the specimens were collected, and in most instances the hydrogen ion concentration was determined within 1 hour. Nitrogen determinations were not made upon a few of the specimens.

Tables I and I A were prepared as were the similar tables in the earlier papers. The results from each specimen of urine were arranged in order of the differences in some factor, and the number of pairs of determinations in which this factor varied was counted. The number of pairs in which the direction of the variation of other factors agreed with that of the independent variable (see the foot-notes to the tables for information as to whether this agreement was studied as a direct or inverse relationship) was

TABLE I

Independent variable		Dependent variable			
Kind.	Variation	Pairs	Reaction	Volume	Ammonia.
			<i>per cent</i>	<i>per cent</i>	Mg per 100 cc <i>per cent</i> Mg per hr.
Volume.	Any.	1,296	60		62
"	More than 5 cc per hr.	1,034	63		64
"	" 10 " "	827	66		67
"	" 20 " "	534	74		73
"	" 50 " "	167	82		63
Reaction.	Any.	1,250		63	60
"	More than 0.1 pH.	1,116		66	63
"	" 0.2 "	1,005		67	64
"	" 0.5 "	757		75	65
"	" 1.0 "	474		78	70

Ammonia concentration decreases and rate of excretion increases as volume increases. Ammonia concentration and rate of excretion increase as acidity increases. Volume decreases as acidity increases.

TABLE I A.

Independent variable.		Dependent variable.					
Kind	Variation	Pairs	Ammonia		Total nitrogen.		Ratio
			Mg per 100 cc per cent	Mg per hr per cent	Mg per 100 cc per cent	Mg per hr per cent	per cent
Volume	Any	968			77	82	58
	More than 5 cc per hr.	783			81	89	60
	" " 10 " "	632			87	5	60
	" " 20 " "	425			92	96	58
Reaction	" " 50 " "	144			97	99	74
	Any.	946			63	41	71
	More than 0.1 pH.	845			64	38	76
	" " 0.2 "	772			67	39	77
Total nitrogen excretion	" " 0.5 "	591			74	34	75
	" " 1.0 "	347			80	33	81
Total nitrogen excretion	Any	989		66			
	More than 50 mg per hr	855		65			
	" " 100 " "	706		65			
	" " 250 " "	372		73			
Total nitrogen concentration	" " 400 " "	159		80			
	Any	983	61				
	More than 0.05 gm per 100 cc	861	66				
	" " 0.1 " 100 "	816	65				
Total nitrogen concentration	" " 0.2 " 100 "	667	70				
	" " 0.5 " 100 "	350	77				

Total nitrogen concentration varies inversely and the rate at which it is excreted, directly, with the volume. Both concentration and rate of excretion of total nitrogen are highest when acidity is greatest. Ratio is highest when acidity is highest and when volume is smallest. Ammonia excretion and concentration vary directly with total nitrogen excretion and concentration.

also counted and the result expressed as percentage of the total pairs. The tables also show the effect of the degree of difference between the values of the independent variable.

The tables make it clear that it is difficult to interpret the effect of any one factor upon the excretion of ammonia, for in several instances there is agreement between these factors themselves. The nitrogen excretion and concentration varied closely with the volume, and the volume and the reaction varied together. Agreement between these two last factors has been found in the three series studied so far, but it seems inadvisable to conclude that variations in both may not be produced by the same cause, for in some cases with achlorhydria rather great changes in volume are not accompanied by marked changes in reaction. The most marked agreement of the ammonia with any of the other factors is that between the ammonia concentration and the reaction. This agreement is not only the closest between ammonia and any of the factors considered as possible independent variables, but it is also the closest agreement between the reaction and any of the urine constituents studied. It seems to the author that the evidence is sufficient to justify the conclusion that there is a causal relationship between the two. It should be noted that in this series the agreement between the ammonia concentration and the reaction is closer than between the ratio ammonia nitrogen: total nitrogen and the reaction. This is different from the findings of Hasselbalch described above.

In order to decide whether there is agreement between ammonia and volume or ammonia and total nitrogen Tables II to IV have been constructed. In Table II only those specimens which showed differences between the reactions of 0.2 pH or less have been included, and the other findings have been analyzed as they were in the tables already described. This table shows that when the reaction was constant the rate of ammonia excretion varied approximately with the volume and the amount of nitrogen excreted, but that the agreement between ammonia and volume was closer than between ammonia and nitrogen. It seems probable that the agreement last named is incidental to the agreement which exists between the rates of nitrogen and water excretion. The agreement between volume and ammonia shown in Table II is not so close as was that shown in the series of Hubbard and Munford; the author believes

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TABLE II.

Reactions vary 0.2 pH or less.

Independent variable			Ammonia. Mg per hr.
Kind	Variation	Pairs	<i>per cent</i>
Volume.	Any.	314	69
"	More than 5 cc. per hr.	239	73
"	" " 10 " " "	167	80
"	" " 20 " " "	85	89
"	" " 50 " " "	36	94
Total nitrogen excretion	Any.	218	62
" " "	More than 50 mg per hr.	190	65
" " "	" " 100 " " "	161	62
" " "	" " 250 " " "	70	70
" " "	" " 400 " " "	26	88

Ammonia excretion varies directly with volume and total nitrogen excretion.

TABLE III.

Volumes vary 10 cc. or less.

Independent variable			Ammonia. Mg per 100 cc.
Kind	Variation	Pairs	<i>per cent</i>
Reaction.	Any.	463	76
"	More than 0.1 pH.	401	79
"	" " 0.2 "	349	83
"	" " 0.5 "	239	90
"	" " 1.0 "	161	94
Total nitrogen concentration.	Any.	336	48
" "	More than 0.05 gm. per 100 cc.	300	49
" "	" " 0.1 " " 100 "	264	51
" "	" " 0.2 " " 100 "	199	53
" "	" " 0.5 " " 100 "	73	55

Ammonia concentration increases as reaction of urine becomes more acid. It varies directly with the total nitrogen concentration.

that the presence in the earlier series of a number of determinations upon cases of achlorhydria may account for the difference. In those cases the reaction of the different specimens was approximately constant, while in the normal subject studied the reactions of successive specimens were often very different, changes during the periods of collection would cause irregularities in the results, and such changes were probably absent in specimens from patients with achlorhydria.

In Table III the results of determinations upon specimens which had approximately the same volume are similarly studied. This table shows approximately the same agreement between reaction and ammonia concentration as did Table I A which included all the results. In the series of Hubbard and Munford agreement was better when specimens which were approximately equal in volume were compared. As there are more results available for comparison in the present series than in the former one it seems probable that the conclusions drawn from them should be considered as more nearly correct, but, on the other hand, there were fewer specimens with large volumes in the present series, so there would not be much difference between conclusions based upon all results and on those in which the volume was constant. Table III shows that the ammonia concentration varies with the reaction rather than with the nitrogen concentration.

In Table IV results from specimens in which the reaction did not vary more than 0.2 pH and the volume or the rate of nitrogen excretion was also approximately the same have been studied. There were only a few determinations which fulfilled these conditions, but the results show clearly that in this series there was better agreement between the volume of fluid excreted and the ammonia than between nitrogen excretion and ammonia. In fact, when the volume and the reaction were both the same there was no relationship between nitrogen and ammonia elimination.

While the method used in studying the results is not one which will show exact agreement between factors—for it only shows whether a particular determination is in its proper place in relationship to other results, and not whether its value is such as would be predicted from a study of other values in the series—still it is well adapted for comparing the effect of two variables on a third, and when the agreement shown between two is close it makes it probable that there is a true relationship between those factors.

TABLE IV.

Reactions vary 0.2 pH or less.

Second constant and variation	Independent variable and variation.	Pairs	Ammonia Mg per hr.
Volume varies 10 cc or less			<i>per cent</i>
" " " "	Total nitrogen varies at all	89	56
" " " "	" " more than 50 mg per hr.	67	57
" " " "	" " " 100 "	48	56
" " " "	" " " 250 "	7	43
Total nitrogen varies 100 mg per hr. or less	Volume varies at all.	53	72
" " " "	" " more than 5 cc. per hr.	31	68
" " " "	" " " 10 "	13	77
" " " "	" " " 20 "	4	75

Ammonia excretion varies directly with the volume and total nitrogen excretion.

In the present series the author believes that the results justify the following conclusions: first, that there was a relationship between the reaction of specimens and the concentration of ammonia which they contained, that the agreement between them was closer than was that between reaction and either the amount of ammonia excreted or the ratio between total nitrogen and ammonia, and that the volume of the specimens had no effect upon the agreement; second, that there was agreement between the rates at which ammonia and fluid were excreted when the reaction was constant; third, that there was no relationship between the rates at which nitrogen and ammonia were excreted which could not be better explained as a relationship between volume and ammonia. These results confirm in general the findings of Hubbard and Munford upon the excretion of ammonia by normal human subjects.

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## SOME CHANGES IN THE COMPOSITION OF BLOOD DUE TO THE INJECTION OF INSULIN.\*

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Studies of the acid-base relations and of the inorganic elements of the blood are of interest in any condition associated with tetany.<sup>1</sup> Characteristic manifestations of large doses of insulin in dogs are: at first, a period of marked depression and muscular weakness which gradually passes into a period of hyperirritability in which the animal may have twitchings or more generalized convulsions.

We had expected to study the alterations in the blood during the latter period, but in our first experiment we took a sample of blood before the onset of hyperirritability. Analyses showed some very interesting changes, so in subsequent experiments the second blood specimen was always taken during the period of depression; if a third was taken, it was obtained before general convulsions supervened but usually during the hyperirritable stage.

\* All the experimental work was done at Washington University School of Medicine.

<sup>1</sup> The term tetany, it seems, should be restricted to conditions of hyperirritability as evidenced by electrical reactions (Denis and von Meysenbug, 1923). Observations upon electrical excitability were not included in these experiments, but the condition of hyperirritability preceding the convulsions was manifested by various twitchings which were elicited or exaggerated by stimuli such as simply tapping the cage.

## EXPERIMENTAL.

*Experimental Procedure.*—The work was done on dogs which had been fasted for 2 days previous to the experiment. Samples of blood were collected under oil from the femoral artery by puncture through the skin. One sample of the blood was centrifuged (under oil) to obtain plasma for the determination of inorganic elements; another was analyzed for oxygen, carbon dioxide, glucose, and lactic acid. Two portions of the whole blood were equilibrated with carbon dioxide tensions, one of which was less and the other more than the value expected for the arterial carbon dioxide tension, and then analyzed for carbon dioxide. From the oxygen and carbon dioxide analyses the arterial pH was computed.<sup>2</sup>

The methods employed in the analytical work were as follows: lactic acid, Clausen (1922); sugar, Shaffer-Hartmann (1921); carbon dioxide and oxygen, Van Slyke and Stadie (1921); and inorganic elements, Briggs (1923).

*Experimental Results.—Alterations Due to Insulin.*—The data obtained from our experiments are summarized in Table I. The changes in each substance determined are shown more clearly in Tables II, III, IV, and V, and Fig. 1.

Leaving the bicarbonate out of consideration for the moment, it is seen that all the inorganic elements with the exception of potassium and phosphate show a remarkable constancy. Both potassium and phosphate show a decided decrease during the period of depression, but in the few experiments in which a sample of blood was taken during the stage of hyperirritability the phosphate had risen to above the control value and the potassium had generally started a return toward the normal.

The partial disappearance of inorganic phosphate,<sup>3</sup> which occurs

<sup>2</sup> The results of seven experiments upon three of the dogs used in this work give an average value of 0.57 cc. of CO<sub>2</sub> carried for 1 cc. of O<sub>2</sub> unsaturation without change in hydrogen ion concentration. The individual values which varied from 0.52 to 0.63 at pH 7.30 are considerably higher than the average value 0.45 obtained on human blood (Doisy, Briggs, Eaton, and Chambers, 1922). The pH of each arterial blood was corrected for oxygen unsaturation by use of the figure 0.57.

<sup>3</sup> Wigglesworth, Woodrow, Smith, and Winter (1923) have published results which show a fall in the inorganic phosphate of the blood of rabbits after insulin with a return toward the normal value upon the injection

during the period in which the glucose is likewise below normal, is possibly in keeping with the views of Embden, Griesbach, and Laquer (1914-15) that phosphate-carbohydrate compounds are concerned in the metabolism of carbohydrate. Our few figures (Table IV) upon whole blood indicate that, notwithstanding the decrease of inorganic phosphate, the acid-soluble phosphate of whole blood does not change; possibly the inorganic phosphate has been used in the synthesis of a compound such as hexose phosphoric acid which has been stored in the cells. The formation of organic phosphate can at most account for but a small fraction of the lost glucose; such a compound may, however, be a transient though important factor in the intermediary metabolism.

Our results on potassium are difficult to interpret; there seems to be a loss from cells as well as plasma.

Contrary to the experiments of Dixon and Pemberton which are cited by Macleod (1923) we find significant decreases of bicarbonate and pH. These changes are to be expected from the increases of lactic acid and are probably to be considered as a direct effect. The increase of lactic acid is of approximately the same magnitude as the decrease of alkaline reserve.

We feel certain that increased muscular activity played no part in the observed production of lactic acid. The most conspicuous outward effect of the insulin at the time of taking the second blood sample was the drowsy, asthenic condition. During this period the respirations seemed to be greater in depth and the rate a little less. A local anoxemia might conceivably have been the cause of the lactic acid production, but the general systemic blood (femoral artery) was well aerated. The oxygen unsaturation was always less than 15 per cent and generally less than 10 per cent.

of glucose. They have found no change in the acid-soluble phosphate. We have performed some experiments upon rabbits, but our results are so contradictory and confusing that we have omitted them from this paper.

Harrop and Benedict (1923) have published a preliminary note in which attention is called to the effect of insulin upon the inorganic phosphate and potassium of the blood. Their work is particularly interesting because of the comparison between the effects of strychnine- and insulin-induced convulsions.



## Dog 4

Aug. 4, 1923	10 00 a.m.	0 119	32 1	3 5	18 3	320	10 9	3 4	598	42 0	10 15 a.m. 10 cc. insulin 386.
	12 30 p.m.	0 107	119 0	1 2	16 3	314	11 3	3 3	576	25 1	12 01 p.m. 10 " 86.

## Dog 5

Aug. 8, 1923	10 45 a.m.	0 121	53 5	3 0	18 3	320	10 0	3 0	612	38 2	7 41	11 15 a.m. 5 cc. insulin 88.
	12 45 p.m.	0 103	73 2	1 3	15 7	325	10 0	2 8	596	27 4	7 335	11 45 " 5 " 88.

## Dog 6

Aug. 13, 1923	10 30 a.m.	0 108	32 1	3 54	18 0	338	10 4	2 3	652	36 2	7 36	10 35 a.m. 8 cc. insulin 88.
	1 45 p.m.	0 072	57 5	0 87	10 4	329	10 0	2 0	612	29 0	7 31	12 00 n. 3 cc. insulin 88.
Aug. 21, 1923	4 40 "	0 064	53 5	3 70	10 7		10 3	2 3	640	36 1		
	10 05 a.m.	0 106	23 2	4 17	22 8		10 0	2 2	661	38 8	7 422	10 25 a.m. 9 cc. insulin X2.
	1 30 p.m.	0 055	20 8	3 10	18 3		10 7	2 2	664	36 9	7 262	11 30 " 10 " X2,
	4 45 "	0 053	25 6	5 92	17 1				647	39 1	7 132	20 units
Aug. 28, 1923	10 30 a.m.	0 084	25 6	5 5	19 4		10 4			38 6	7 380	
	1 30 p.m.	0 035	30 3	2 2	16 9		10 0			37 7	7 309	

## Dog 7

Aug. 15, 1923	10 28 a.m.	0 100	61 4	2 4	17 1	334	9 8		624	36 7	7 283	10 40 a.m. 3 cc. insulin 88.
	1 40 p.m.	0 062	101 0	0 8	9 5	320	10 7		602	25 6	7 127	12 05 p.m. 3 " 88.
Aug. 16, 1923 " 23, 1923	3 40 "	0 046	83 9	2 9			10 7		627	32 8	7 000	
	11 00 a.m.	0 130	23 8				9 1		622	38 7	7 302	
	9 10 "	0 112	54 2	2 6	21 0	330	11 0	2 2	648	40 5	7 32	
	12 18 p.m.		50 5	3 0	20 3	322	10 7	2 1	641	40 8	7 28	Control.

TABLE II

*Characteristic Changes Produced by Insulin.*

Glucose *			Lactic acid *			Inorganic phosphorus †			Potassium †		
Before	After	Later	Before	After	Later	Before	After	Later	Before	After	Later
mg	mg	mg	mg	mg	mg	mg	mg	mg	mg.	mg.	mg.
108	70		20 5	32 9		1 5	1 0		19 5	15 4	
131	67		19 8	62 0		2 6	0 8		19 9	11 7	
118	63	80	37 6	93 0	34 6	2 4	1 0	3 6	18 6	13 1	16 6
114	74	62	21 5	41 7	25 2	2 9	0 8	4 2	18 0	13 2	15 4
119	56					1 7	1 6		21 0	20 0	
119	107		32 0	119		3 5	1 2		18 3	16 3	
121	103		53 5	73 2		3 0	1 3		18 3	15 7	
108	72	64	32 1	57 5	53 5	3 5	0 9	3 7	18 0	10 4	10 7
106	55	53	23 2	20 8	25 6	4 2	3 1	5 9	22 8	18 3	17 1
84	35		25 6	30 3		5 5	3 2		19 4	16 9	
100	62	46	61 4	101 0	83 9	2 4	0 8	2 9	17 1	9 5	
Average	112	70	33 5	66 8		3 0	1 3		19 2	14 6	

\* Figures for glucose and lactic acid are in mg per 100 cc of whole blood.

† Figures for inorganic phosphorus and potassium are in mg. per 100 cc. of plasma.

TABLE III

*Elements Which Showed No Significant Change after Insulin.*

Sodium		Chlorides		Calcium		Magnesium	
Before	After	Before	After	Before	After	Before	After
<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
321	326	562	612	10 9	10 5	2 1	1 9
324	333	590	613	10 7	11 4	2 3	2 2
337	320	641	612	10 3	10 3	2 1	2 0
341	339	618	633	11 6	12 1	2 3	2 1
316	329	602	618	11 3	11 9	2 3	2 3
320	314	598	576	10 9	11 3	3 4	3 3
320	325	612	596	10 0	10 0	3 0	2 8
338	329	652	612	10 4	10 0	2 3	2 0
		661	664	10 0	10 7	2 2	2 2
334	320	624	602	9 8	10 7		
Average.... 329	326	615	615	10 6	10 8	2 4	2 3

Although our experimental data are insufficient for the establishment of a theory of the effect of insulin upon the carbohydrate metabolism, it may be permissible to indulge in a few speculations and an endeavor to correlate some other observations with ours. In the first place there is not a great deal of positive evidence in favor of any of the various hypotheses concerning the disappearance of the blood sugar (see Dale, 1923) of normal animals.

The experiments of Dudley, Laidlaw, Trevan, and Boock (1923) indicate that the sugar does not disappear from the blood

TABLE IV.  
*Phosphates on Blood and Plasma*

Date	Blood				Plasma			
	Acid-soluble		Inorganic		Acid-soluble		Inorganic	
	Before	After	Before	After	Before	After	Before	After
1923	mg	mg	mg	mg	mg	mg	mg	mg
Aug. 18	27 3	27 3	2 1	1 1	2 2	1 2	2 4	1 0
" 21	30 0	30 4	3 92	2 90	4 28	3 43	4 17	3 10
" 28	25 5	25 0	4 5	2 7	5 6	2 6	5 5	2 2

TABLE V  
*Potassium on Blood and Plasma*

Date	Blood		Plasma	
	Before	After	Before	After
1923	mg	mg	mg	mg
Aug. 18	28 9	30 4	16 1	13 1
" 21	35 1	31 5	22 8	18 3
" 28	30 5	26 9	16 9	15 2

because of increased combustion. Kellaway and Hughes (1923) have found increases in respiratory quotients, but do not believe that the oxygen utilization is sufficient to account for the glucose that has disappeared. Lyman, Nicholls, and McCann (1923) have shown that small doses of insulin given to normal men increase both the respiratory quotients and heat production. Although Kellaway and Hughes do not so interpret their results it seems probable that insulin does cause a slight increase in the rate of combustion of carbohydrate.



With regard to the other hypotheses of conversion to glycogen or to fat, the paper of Dudley and Marrian (1923) offers direct

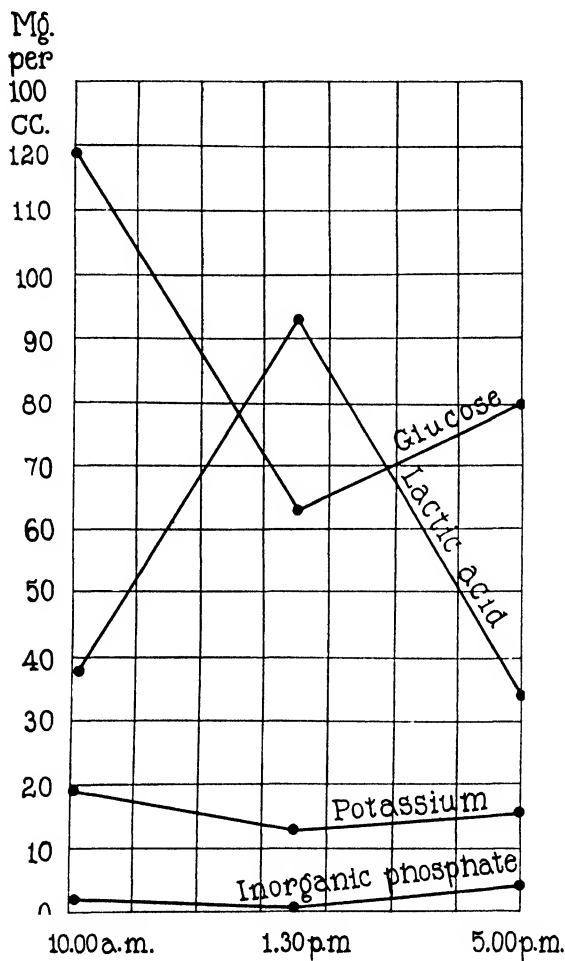
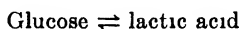


FIG. 1. Changes found in the experiment of Aug. 18, 1923.

contradiction. They have found no change in the liver fat and an actual decrease in the glycogen content of liver and muscle.

There seems to be no doubt from our results that the disappearance of sugar is due to the formation of lactic acid. The

trend of biochemical opinion is to lay considerable stress upon the reversible reaction



Embden and Isaac (1917) have shown that the liver from a diabetic animal appears to have lost the power to form lactic acid from glucose and that lactic acid is converted to glucose.

Our belief is that insulin influences the reversible reaction in the direction of lactic acid and that glucose is formed in diabetic animals because the influence is lacking. That lactic acid is subsequently disposed of in part by oxidation is indicated by the slight rises in oxygen consumption referred to above.

We would like to point out that if the lactic acid rise occurs in all the tissues as well as blood the  $\text{CO}_2$  liberated is sufficient to raise appreciably the respiratory quotients. Such a condition must be taken into consideration in the study of respiratory data on normal animals following the use of insulin.

#### CONCLUSION.

Insulin causes a decrease in the concentration of glucose, inorganic phosphate, and potassium in the blood of normal animals. There is a parallel increase in lactic acid apparently formed from glucose under the influence of insulin.

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## SOME CHEMICAL REACTIONS OF THE SUBSTANCE CONTAINING INSULIN.\*

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(Received for publication, November 8, 1923.)

The pancreatic substance containing insulin<sup>1</sup> used in the experiments recorded below was prepared from the pancreas of hogs according to the method developed<sup>2</sup> by Mr. George B. Walden of the Lilly Research Laboratories, and which has been published by the Council of Pharmacy and Chemistry of the American Medical Association.<sup>3</sup>

This experiment, which is an extension of Mr. Walden's work, was started at the suggestion of Dr. G. H. A. Clowes with the hope of isolating the physiologically active substance as a compound possessing a definite chemical constitution, or at least of determining some characteristic reaction peculiar to the active principle. Though neither of these results has been achieved it was thought that the somewhat disconnected data secured might be of interest.

A solution of insulin, thus prepared, gives almost all the protein tests, and consists chiefly of proteoses, together with some peptones, peptides, and amino-acids. Different lots prepared

\* A report of certain phases of the chemical investigation of insulin in which this laboratory has been engaged since October 1, 1922. This paper was read before the Medicinal Products Section of the American Chemical Society at the 1923 Milwaukee meeting. At our request the paper was released from publication in the *Journal of the American Chemical Society*.

<sup>1</sup> All the material used was manufactured by Eli Lilly and Company under the trade name "Iletin."

<sup>2</sup> This method is a development of the method discovered by Banting, Best, Collip, and Macleod (Banting, F. G., Best, C. H., Collip, J. B., and Macleod, J. J. R., *Tr. Roy. Soc. Canada*, 1922, xvi, 1)

<sup>3</sup> Iletin (Insulin—Lilly), *J. Am. Med. Assn.*, 1923, lxxx, 1851.

in the same manner will show different proportions of proteose and its degradation products per unit.<sup>4</sup>

Protein precipitants such as tungstic, phosphotungstic, nitric, trichloroacetic, tannic, picric, and *m*-phosphoric acids; absolute alcohol; sodium, zinc, and ammonium sulfate, etc., carry down the active substance in the precipitate formed, leaving an inactive filtrate containing polypeptides and amino-acids. Regardless of whether the precipitating agent formed a definite compound with the insulin or whether it was merely a salting out effect, usually the entire amount of active material can be recovered from the precipitate.

By repeated precipitation at the isoelectric point or with trichloroacetic acid it is possible to secure an active preparation which contains a minimum amount of nitrogen and which is practically free from non-protein nitrogen. While reprecipitation tends to free the insulin from a part of the inert substances, if repeated too often it considerably weakens the active material so that in the final product we do not find an increase in the percentage of activity corresponding to the quantity of impurity removed. Inactivation has occurred during the manipulation, since the filtrate is inactive. 1 cc. of purified concentrated insulin, containing 100 units,<sup>5</sup> gives a negative Molisch reaction (carbohydrate); a positive biuret reaction (protein); a faint ninhydrin reaction ( $\alpha$ -amino-acid group); a negative Hopkins-Cole reaction (tryptophane); a negative Ehrlich's *p*-dimethylaminobenzaldehyde reaction (indole derivatives); a faint Millon's reaction (tyrosine); a positive Ehrlich's diazo reaction (tyrosine

<sup>4</sup> The physiological standardization of the insulin used in this report was carried out on rabbits, the unit being the provisional clinical unit formerly used by the Insulin Committee of the University of Toronto. This unit was one-third of the minimum amount of insulin which on subcutaneous injection into fasted 2 kilo rabbits lowered the blood sugar to the convulsive level, 0.045 per cent, within 2 to 5 hours in 60 to 70 per cent of the animals employed. In no case have the results been based on data secured from the injection of two or three rabbits alone.

<sup>5</sup> In making tests on insulin, it is necessary that the amount of organic material in solution is sufficiently large to come within the range of the sensitiveness of the reaction. A 1 cc. sample containing a few units does not give many of the above tests simply because the concentration of the reacting groups is too small.

and histidine);<sup>6</sup> a positive xanthoproteic reaction (benzene nucleus); a positive reaction for cystine and tyrosine by Folin and Looney's method;<sup>7</sup> a positive test for reduced sulfur after boiling with NaOH; no color with sodium nitroprusside (SH groups); no phosphorus after fusion; and after hydrolysis, a negative Wheeler-Johnson reaction (uracil and cytosine) and no precipitate with ammoniacal silver nitrate (purines).<sup>8</sup>

Though the above reactions were run quantitatively whenever possible, no correlation could be secured between the intensity of any of these reactions and the physiological activity in the most highly purified products. The destruction of the physiological activity does not affect any of the above reactions.

It has not been possible to concentrate insulin by repeated precipitation with protein precipitants or at the isoelectric point beyond the point of 0.006 mg. of nitrogen per unit. The percentage of nitrogen shows considerable variation, with the majority of the preparations having a nitrogen content of 17 to 20 per cent. While the nitrogen determinations in the course of purification were carried out by the micro Kjeldahl method,<sup>9</sup> the final evaluations were determined by combustion. The composition of a number of samples is given in Table I.

From the data of Table I it is apparent that precipitation methods are inadequate to purify insulin to the point of securing a substance of constant composition. The use of the same methods of purification on different lots yields products showing a marked variation in the C, H, and N content. It is probable that we are dealing with a complex mixture of closely related substances whose composition during the process of reprecipitation tends to become constant. Factors, as yet uncontrollable, determine the proportion of these related substances in the gland extract.

<sup>6</sup> The destruction of tyrosine by nitration (Brunswick, H., *Z. physiol. Chem.*, 1923, cxxvii, 268) allows a positive test for histidine. Histidine was detected after nitrating insulin.

<sup>7</sup> Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 421.

<sup>8</sup> Best and Macleod (Best, C. H., and Macleod, J. J. R., *J. Biol. Chem.*, 1923, lv, p. xxix), Doisy, Somogyi, and Shaffer (Doisy, E. A., Somogyi, M., and Shaffer, P. A., *J. Biol. Chem.*, 1923, lv, p. xxxi), and Dudley (Dudley, H. W., *Biochem. J.*, 1923, xvii, 376) have reported on some chemical reactions of insulin. Practically all the above tests were made prior to the publication of these papers.

<sup>9</sup> Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

Purified insulin is soluble in dilute acids and alkalis, but is insoluble at a pH of 4.7 to 5.0.<sup>10</sup> Insulin is unstable toward alkali, but is relatively stable toward acid. When inactivated

TABLE I

Sample No	Description	C*	H*	N*	Ash	Organic solids	N
		per cent	per cent	per cent	mg per unit	mg per unit	mg per unit
Z-1	"Crude insulin," dried precipitate from 93 per cent alcohol precipitation	42 60	7 47	17 44	16 50	2 2200	0 3870
Z-2		40 19	8 70	18 39	17 90	0 5460	0 1000
725,679		39 25	6 45	13 52	19 80	1 7700	0 2390
729,715	"Crude insulin" reprecipitated at isoelectric point until N per unit was constant	51 48	12 24	15 85	1 90	0 0491	0 0078
729,719		51 15	7 77	21 85	2 45	0 0585	0 0127
729,719	Last filtrate from No 729,719			21 67	23 50	0 1290	0 0279
51,023	"Crude insulin" precipitated at isoelectric point and with trichloroacetic acid			18 02	4 00	0 0499	0 0090
51,023Z	Reprecipitation of No 51,023 at isoelectric point and with trichloroacetic acid.	41 12	8 68	17 95	3 66	0 0613	0 0109
62,923	"Crude insulin" precipitated at isoelectric point and with trichloroacetic acid	41 32	10 00	19 76	2 60	0 0486	0 0096
71,223	Purified in the same way as No. 62,923	36 16	6 13	20 06	3 20	0 0840	0 0168
72,723	Purified in the same way as No. 62,923	38 83	6 41	18 43	0 00	0 0337	0 0061

\* C, H, and N determinations are based on organic solids.

<sup>10</sup> Crude insulin, which is water-soluble, can be purified and the entire activity secured in a fraction, which is insoluble in water. Witzemann and Livshis (Witzemann, E. J., and Livshis, L., *J. Biol. Chem.*, 1923, lvi, 425) postulate a peptone-polypeptide type and a protein type of insulin based on the difference in solubility of crude and purified insulin.

by heating with alkali or with acid no increase in  $\alpha$ -amino-acid groups could be detected with the ninhydrin test, though in the latter case some hydrolysis must have occurred. It would appear, then, that only a slight disintegration of a complex molecule is necessary to destroy the insulin. Possibly the alkali racemizes<sup>11</sup> an essential group in the structure while the acid acts by slow hydrolysis. Numerous unsuccessful attempts have been made to reactivate the insulin destroyed by a brief warming in an alkaline medium through warming with acid.

It is soluble in acidified absolute alcohol, but not in neutral or alkaline alcohol. But slight purification could be effected by this means since the protein accompanying the active fraction is likewise soluble in acid, but not in neutral or alkaline alcohol. It is insoluble in ether, benzene, and chloroform.<sup>12</sup>

Hiller and Van Slyke<sup>13</sup> have shown that trichloroacetic acid in concentrations below 5 per cent in serum allows some proteose and all the intermediate products such as peptones and peptides to go through into the filtrate. Since 95 per cent of the activity of insulin is precipitated by a 2.5 per cent concentration of trichloroacetic acid, it would appear that the activity does not reside in the lower fragments of the protein molecule. Fractional precipitation of purified insulin with trichloroacetic acid or at the isoelectric point does not materially vary the amount of solid or nitrogen per unit in each fraction.

Quite early in the investigation of insulin it was necessary to determine the stability of insulin to all proteolytic enzymes. It was found that insulin was rapidly destroyed by pepsin, papain, trypsin, and erepsin. 2 hours incubation of 3,000 units of insulin with 10 mg. of trypsin completely destroyed four-fifths of the activity.<sup>14</sup> Adequate controls were run in each case. An examination of the products of digestion secured just at the point of complete inactivation is in progress.

<sup>11</sup> Insulin, in common with all unconjugated proteins, is levo-rotatory.

<sup>12</sup> Widmark (Widmark, E. M. P., *Biochem J.*, 1923, xvii, 669) discusses the solubility of a crude insulin which contains one rabbit unit per 20 to 25 mg. of solids.

<sup>13</sup> Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liii, 253.

<sup>14</sup> Banting and Best (Banting, F. G., and Best, C. H., *J. Lab. and Clin. Med.*, 1921-22, vii, 251, and private communication) found trypsin and pepsin destructive, and Witzemann and Livshis (Witzemann, E. J., and Livshis, L., *J. Biol. Chem.*, 1923, lvii, 425) report on the destruction of insulin by pepsin, trypsin, and papain



Insulin is very readily adsorbed by kaolin, charcoal, Lloyd's reagent, etc., and the amount adsorbed being, in the case of Lloyd's reagent, proportional to proteose adsorbed. Positive and negative colloids precipitate insulin along with the proteose.

During the prolonged dialysis of purified insulin in parchment paper or collodion sacs, part of the physiologically active substance passed into the dialysate. In most cases only 10 to 20 per cent of the activity was found in the dialysate, but in one set of experiments half of the activity passed through the membrane. The nitrogen content of the dialysate per rabbit unit was 0.002 mg. (micro Kjeldahl method). Ehrlich's diazo reaction has the same intensity per unit as had the original preparation, while the sulfur content as shown by the lead acetate test after boiling with NaOH was decidedly less. Cystine and tyrosine were present, and the other protein reactions present in the original substance were found. This insulin preparation was the purest we have obtained and we regret that there has not yet been a sufficient amount prepared to determine the C, H, and N content.

The Van Slyke method shows no amino-acid in purified insulin samples containing 20 mg. of solids. After complete acid hydrolysis the same sized sample has 70 to 75 per cent of its nitrogen as  $\alpha$ -amino-acid nitrogen. The melanin and amide nitrogen constitute 7 to 10 per cent, the diamino-acid fraction 35 per cent, tyrosine 3.6 per cent, and cystine 3.2 per cent of the total nitrogen. Further investigation is in progress to determine whether there are any constituents other than amino-acids present.

Insulin is readily destroyed by oxidation with very dilute solutions of hydrogen peroxide or potassium permanganate.

Reducing agents such as sodium bisulfite, sulfur dioxide, hydrogen, and stannous chloride destroy the activity. All attempts to reactivate reduced insulin by oxidation have failed.

From the above data we can only conclude that the pancreatic substance containing insulin appears to be a complex mixture of proteoses, which give typical protein reactions and from which it has been as yet impossible to isolate a simple substance or to detect a chemical reaction that is characteristic of the physiologically active constituent. Further research must determine whether the active principle is a proteose or is merely intimately associated with a proteose fraction.

# THE ISOLATION AND IDENTIFICATION OF RUTIN FROM THE FLOWERS OF ELDER (*SAMBUCUS* *CANADENSIS* L.).\*

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Washington, and the Laboratory of John Uri Lloyd, Cincinnati, Ohio )

(Received for publication, November 19, 1923 )

A yellow pigment isolated from the white flowers of the elder (*Sambucus canadensis* L.) was briefly described in 1920 and tentatively called "eldrin."<sup>1</sup> At that time no attempt was made to establish the exact identity or to determine its composition. These details are supplied in the present paper which embodies the results of a more complete investigation of the pigment.

## *Preparation of Eldrin*

The elder flowers for this investigation were collected in June near Cincinnati, Ohio. The flowers, freed as far as possible from stems, were dried in the shade, finely ground, and exhaustively extracted by percolation with 95 per cent alcohol. The pale green alcoholic extract was evaporated to a small volume and then diluted with a large volume of hot water. After filtration of the hot solution, most of the alcohol was removed by evaporation. From the remaining solution the crude pigment separated after several days. This was collected on a Buchner funnel, washed, and dried. By this method, 326 gm. of impure eldrin were obtained from 22.7 kilos of fresh elder flowers.<sup>2</sup> The impure

\* The writers wish to acknowledge the help received in this investigation from Mr. Paul Williams

<sup>1</sup> Lloyd, J. U., Plant constituents, *Eclectic Med. J.*, 1920, lxxx, 591

<sup>2</sup> A quantitative approximation of the eldrin content of air-dried elder flowers was made by extracting 233 gm., first with petroleum ether and ether and subsequently with 95 per cent alcohol, until the final extractions no longer gave a test for flavonol. The alcoholic extract was evaporated

pigment was purified by extraction with successive small quantities of cold 95 per cent alcohol and repeated crystallization from hot water, dilute acetic acid (1 per cent), aqueous pyridine, and finally hot water. The substance was thus obtained in the form of fine, very dense, fan-shaped tufts of silky crystals (Fig. 1) which closely resembled those of rutin from *Eschscholtzia* which is shown for comparison in Fig. 2. The color of the anhydrous compound was primrose-yellow; and the streak, pale-green yellow. The substance was almost insoluble in cold water, and

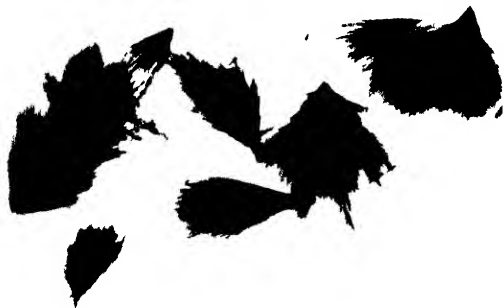


FIG 1 Eldrin, crystallized from hot water by dissolving 0.17 gm. in 30 cc. of water ( $\times 80$ ).

sparingly soluble in hot water, but easily soluble in alcohol and pyridine. Its aqueous solution gave a dark green color with ferric chloride, and yielded an orange-yellow precipitate with lead acetate. Alkalies intensified the orange-yellow color of an aque-

to small bulk and then treated with 3 volumes of ether whereby the impure pigment separated gradually. By repeated crystallization from hot water 1.63 gm. of air-dried eldrin were obtained in a pure state, and a further quantity of 0.16 gm. of slightly impure pigment was obtained from the mother liquors. The air-dried flowers therefore contain about 0.77 per cent of eldrin.

ous solution. A sample of the compound dried over sulfuric acid sintered at  $170^{\circ}$ , and melted indefinitely between  $180$  and  $200^{\circ}$ . The results of combustion of two samples of the material are given below. The first sample (a) was dried in a vacuum desiccator over phosphorus pentoxide for about 3 months, the second sample (b) was dried to constant weight by heating at  $155^{\circ}$ .

(a) 0.1393 gm  $\cdot$  0.0637 gm  $\text{H}_2\text{O}$  and 0.2702 gm  $\text{CO}_2$   
 (b) 0.1139 " : 0.0510 " " " 0.2219 " "

Found. C 52.90, 53.13, H 5.12, 5.02  
 Rutin,  $\text{C}_{27}\text{H}_{30}\text{O}_{16}$ , requires C 53.10, H 4.95



FIG 2 *Eschscholtzia* rutin, crystallized from hot water by dissolving 0.17 gm in 30 cc of water ( $\times 80$ ).

The empirical composition of the substance therefore agrees with that of rutin.

#### *Hydrolysis of Eldrin.*

##### *Formation and Identification of Quercetin.*

A portion of the compound was boiled an hour with approximately 5 per cent sulfuric acid. After hydrolysis, the mixture was set aside overnight. The precipitate which formed was

collected and washed with cold water. It was then repeatedly extracted with small quantities of boiling water, and finally purified by crystallization from dilute alcohol. It formed a glistening mass of yellow needles which were practically insoluble in water, but readily soluble in alcohol. They dissolved in alkaline solutions with a deep yellow color. With alcoholic ferric chloride the compound gave a dark green color. Analysis of the substance, dried in a vacuum at  $120^{\circ}$ , showed that it agreed in composition with quercetin.

0.1127 gm. 0.0349 gm.  $H_2O$  and 0.2453 gm.  $CO_2$

Found C 59.36, H 3.47

Quercetin,  $C_{15}H_{10}O_7$ , requires C 59.59, H 3.34

The identity of this compound with quercetin was confirmed by the formation of an acetyl derivative, which was prepared by boiling the substance for an hour with an excess of acetic anhydride and anhydrous sodium acetate. The reaction mixture was subsequently poured into water and set aside overnight. The acetyl derivative was recrystallized from 95 per cent alcohol. The crystals formed a felty mass of colorless needles. These were insoluble in water and sparingly soluble in cold alcohol. They melted at  $194-196^{\circ}$ . When mixed with penta-acetylquercetin derived from quercitrin, there was no depression in the melting point. An analysis of the acetyl derivative, dried at  $155^{\circ}$ , showed it to be identical in composition with penta-acetylquercetin.

0.1283 gm. 0.0470 gm.  $H_2O$  and 0.2759 gm.  $CO_2$

Found C 58.64, H 4.10

Acetylquercetin,  $C_{15}H_8O_7(C_2H_3O)_5$ , requires C 58.57, H 3.93.

From the penta-acetylquercetin, quercetin was quantitatively regenerated in duplicate samples by dissolving the anhydrous acetyl derivative in glacial acetic acid with the addition of a few drops of sulfuric acid and boiling a short time. After the addition of water the mixtures were set aside 24 hours and filtered through Gooch crucibles. The residues were washed with water, and dried at  $130^{\circ}$ .

0.1942 gm.: 0.1137 gm. quercetin

0.3452 " : 0.2025 " "

Found  $C_{15}H_{10}O_7$  58.55, 58.66

Acetylquercetin,  $C_{15}H_8O_7(C_2H_3O)_5$ , requires  $C_{15}H_{10}O_7$  58.98.

*Formation and Identification of the Sugars.*

In order to identify the sugars formed by the hydrolysis of eldrin, the acid filtrate remaining after the removal of quercetin from the hydrolysis mixture was boiled with barium carbonate and the barium sulfate formed was removed by filtration. The filtrate was then concentrated on the steam bath and heated with sodium acetate and phenylhydrazine until a separation of osazones occurred. The osazones were separated by means of their differential solubility in acetone, and purified by crystallization from alcohol with the addition of aqueous pyridine. By this method, glucosazone, melting at 205–206°, and rhamnosazone, melting at 180–181°, were obtained. They yielded the characteristically different crystals which have already been described in connection with work on rutin from *Eschscholtzia*.<sup>3</sup>

An attempt was made to determine quantitatively the quercetin yielded by the decomposition of pure anhydrous eldrin on the assumption that the eldrin molecule contains 1 molecule of glucose and 1 of rhamnose. The hydrolysis was accomplished by boiling a sample of eldrin  $\frac{1}{2}$  hour under a reflux condenser with approximately 5 per cent sulfuric acid. The solution was left overnight. The precipitated quercetin was collected in a weighed Gooch crucible, washed, and dried at 130°. The yields are given below.

0.4065 gm : 0.2069 gm quercetin.

0.4957 " : 0.2514 " "

Found  $C_{15}H_{10}O_7$  50.89, 50.71.

Rutin,  $C_{27}H_{30}O_{16}$ , requires  $C_{15}H_{10}O_7$  49.50.

It will be seen from these data that eldrin yields an average of 50.78 per cent of quercetin after hydrolysis, whereas the theoretical yield should be only 49.50 per cent. A similar discrepancy has been observed by other investigators between the theoretical proportions of the decomposition products of simple glucorhamnosides of flavonols and anthocyanidins and the quantities actually found. For example, according to a recalculation by Willstätter and Zollinger<sup>4</sup> of data obtained by Schmidt and his

<sup>3</sup> Sando, C. E., and Bartlett, H. H., Rutin, the flavone pigment of *Eschscholtzia californica* Cham., *J. Biol. Chem.*, 1920, xli, 495.

<sup>4</sup> Willstätter, R., and Zollinger, E. H., Über die Farbstoffe der Kirsche und der Schlehe, *Ann. Chem.*, 1916, ccccxii, 164.

students the quantities of rhamnose recovered by hydrolysis of rutin are too low. Furthermore, Willstätter and Zollinger in a determination of cyanidin chloride (anthocyanidin chloride), formed by hydrolysis from keracyanin (gluco-rhamnoside), obtained an average yield of 57.3 per cent instead of 54.0 per cent which theory requires.

Three possible explanations suggest themselves to account for these discrepancies: (1) that the glucoside is incompletely hydrolyzed by the method employed and the recovered pigment is thus contaminated by the unaltered parent substance; (2) that the glucoside before hydrolysis contains a trace of free pigment; and (3) that the glucoside contains as impurity a small quantity of a second compound, which is probably a monoglucoside and difficult to remove. These three possibilities were examined with reference to eldrin. In order to test the first possibility, determinations were made by boiling one sample (a) for 1 hour with 5 per cent sulfuric acid and by boiling another sample (b) the same length of time with 10 per cent hydrochloric acid. The results, which are given below, indicate that complete hydrolysis of eldrin occurs when it is boiled about 1 hour with 5 per cent sulfuric acid.

(a) 0.9841 gm : 0.4994 gm quercetin  
 (b) 0.6008 " : 0.3042 " " "  
 Found  $C_{18}H_{10}O_7$  50.74, 50.63

With respect to the second possibility, it should be mentioned that free quercetin is more soluble in ether than its glucosides, consequently traces of this compound would be removed by long extraction with ether. A sample of eldrin was therefore extracted with ether in a Soxhlet apparatus for 10 hours. The compound was dried in a vacuum desiccator at 145° and hydrolyzed with the following results.

0.4040 gm : 0.2055 gm quercetin  
 0.4512 " : 0.2293 " "  
 Found  $C_{18}H_{10}O_7$  50.86, 50.82.

It is evident from these results that the high quercetin yields obtained when eldrin is hydrolyzed cannot be traced to the presence of free quercetin.

Testing of the third possibility was accomplished by fractional extraction of a new sample of eldrin with water, the quantity of each portion being so regulated that it dissolved approximately one-fourth of the total sample of pigment. After each solution had been boiled, filtered, and cooled, the deposits were collected. The fractions of material so obtained were air-dried. The water of crystallization was determined in each case. Anhydrous samples, dried at 150° in a vacuum, were then hydrolyzed to determine quercetin yields. These are given in Table I.

TABLE I.

Fraction	Water of crystallisation			Quercetin.		
	1	2	Average	1	2	Average
a	10 18	10 47	10 38	50 82	51 05	50 94
b	9 75	10 13	9 94	51 07	50 83	50 95
c	9 38	9 85	9 63	50 68	50 86	50 77
d	9 08	9 28	9 18	49 49	50 01	49 75

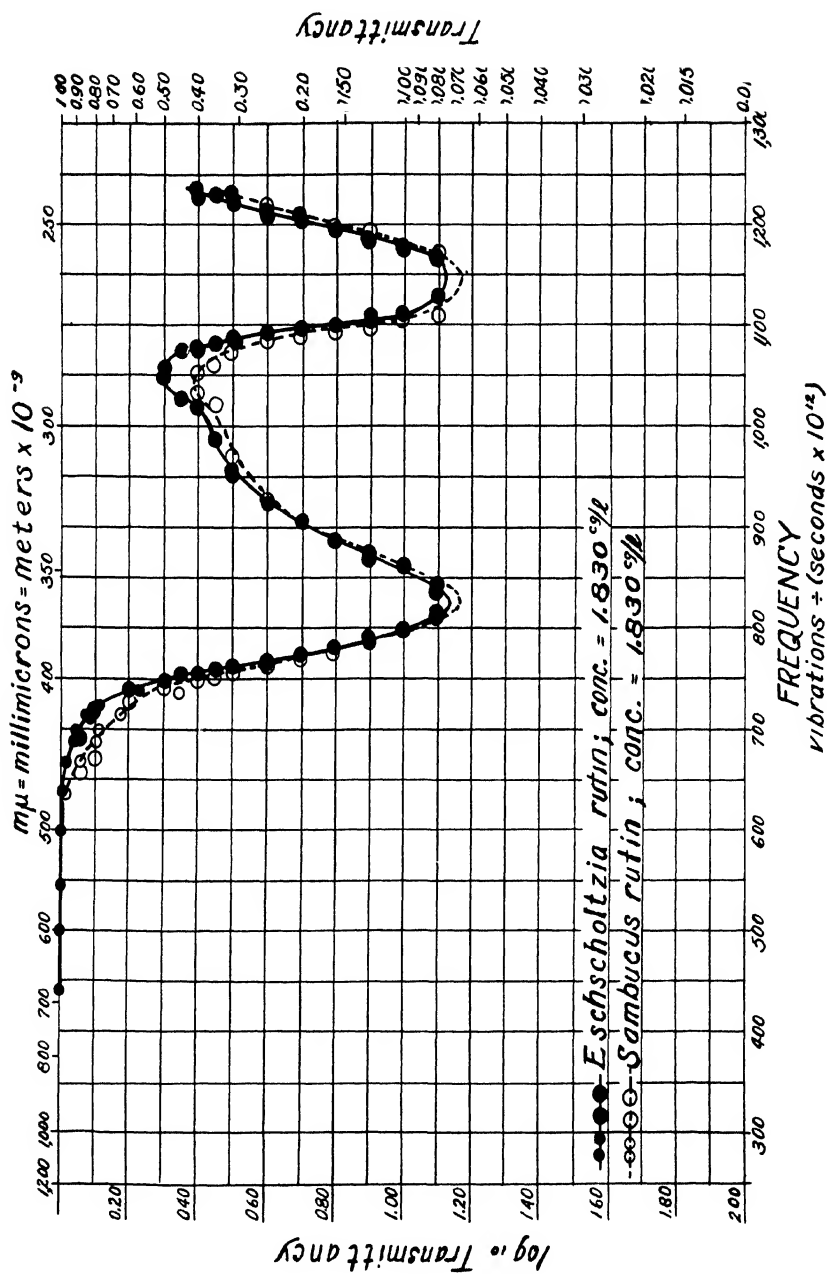
From these results it seems evident that eldrin was mixed with a small quantity of another compound which is very difficult to eliminate. Fraction d appeared to be the purest, although even in this case the figures indicate the presence of some impurity. Pure hydrated rutin,  $C_{27}H_{30}O_{16} \cdot 3H_2O$ , requires 8.13 per cent of water and pure anhydrous rutin,  $C_{27}H_{30}O_{16}$ , requires 49.50 per cent of quercetin.

#### *Spectral Transmittancy of Eldrin.*

In order to compare the spectral transmittancy of eldrin with that of authentic rutin from *Eschscholtzia*, samples of the two pigments were separately dissolved in absolute alcohol. The solutions were made to the same molecular concentration; that is, 1.830 cg. per liter, or  $M/300,000$ . Although for reasons mentioned above it is extremely difficult to obtain eldrin free from traces of impurity, it will be seen from Fig. 3<sup>5</sup> that there is close agreement of eldrin transmittancy with that of rutin.

<sup>5</sup> The curves were kindly prepared by the U. S. Bureau of Standards. The measurements in both the visible and ultra-violet regions of the spectrum were made by Mr. H. J. McNicholas, to whom we wish to express our utmost appreciation.





## SUMMARY.

From the foregoing work it appears that purified eldrin has the same empirical composition as rutin; that upon hydrolysis it yields quercetin, glucose, and rhamnose; that the quantity of quercetin obtained, with a slight difference shown to be caused by the presence of an impurity, agrees with the quantity theoretically expected from rutin; and that the spectral transmittancy of eldrin agrees with that of authentic rutin from *Eschscholtzia*. The conclusion is therefore justified that eldrin, the yellow pigment from the flowers of *Sambucus canadensis* L., is rutin.



## STUDIES OF THE CARBON DIOXIDE ABSORPTION CURVE OF HUMAN BLOOD.

### IV. THE RELATION OF THE HEMOGLOBIN CONTENT OF BLOOD TO THE FORM OF THE CARBON DIOXIDE ABSORPTION CURVE.

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In 1917 Hasselbalch (1), in an article entitled "Über die 'Acidotischen Konstitution' des Neugeborenen," presented carbon dioxide absorption curves from the blood of patients exhibiting various degrees of anemia. He showed that the slope of the absorption curve varied with the hemoglobin content of blood. The same relation was pointed out by Barr and Peters (2), and by Warburg (3). The experimental material presented by these workers indicated that hemoglobin was the chief, but not the sole, determinant of the slope of the absorption curve; but was too scanty to permit the quantitative estimation of the relative value of this determinant. This paper deals with an attempt to evaluate the relation of the hemoglobin content of human blood to the slope of the carbon dioxide absorption curve.

In the second paper of this series (4) there was presented a series of twenty-six experiments in which the  $\text{CO}_2$  content of blood was determined at 20, 40, and 80 mm. of  $\text{CO}_2$  tension at  $38^\circ\text{C}$ . In the first paper (5) is another series of twelve experiments in which the  $\text{CO}_2$  content of both blood and its "true" plasma was determined at 30 and 60 mm. of  $\text{CO}_2$  tension. To the latter series have been added ten more similar experiments. The data thus obtained offer a means of studying the relation of the hemoglobin content of human blood to the slope of the carbon dioxide absorption curve. In order to render all the experiments comparable  $\text{CO}_2$  values at 30 and 60 mm. of  $\text{CO}_2$  tension have been calculated

for all the three point experiments and from these values the buffer value  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  (see Van Slyke (6)) was obtained.

The method of calculation employed was as follows. By means of the logarithmic relation described in the preceding paper of this series (7) the CO<sub>2</sub> content at 30 mm was calculated from CO<sub>2</sub> contents observed at 20 and 40 mm and the CO<sub>2</sub> content at 60 mm from the 40 and 80 mm values. This probably introduces less error than would the use of an average slope obtained from the three points. The bicarbonate was then calculated for each point to give  $\Delta[\text{BHCO}_3]$ . In the estimation of pH a constant  $\text{pK}_1$  of 6.1 was assumed. This is, of course, improper, but substitution of corrected  $\text{pK}_1$  values does not alter the results significantly. In the two-point experiments the CO<sub>2</sub> content of the whole blood at 40 mm was also calculated by means of the logarithmic relation.

The results are shown in Table I. The experiments are arranged in order of ascending oxygen values, which are given in Column 1. Column 2 gives the CO<sub>2</sub> content at 40 mm. of CO<sub>2</sub> tension. As the CO<sub>2</sub> tension is constant this is an indication of both bicarbonate content and pH. Column 3 shows the arithmetical difference between the CO<sub>2</sub> content at 60 mm. and that at 30 mm., which we shall designate  $\Delta[\text{CO}_2]_{60-30}$ ; Column 5, the ratio  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  and the remaining two columns give  $\Delta[\text{CO}_2]_{60-30}$  and  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  of the "true" plasma. In Chart 1, I and II show the relation of oxygen capacity to  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  and to  $\Delta[\text{CO}_2]_{60-30}$ , respectively. The degree of scattering is not appreciably greater in one than in the other, but is considerable in both.<sup>1</sup> Further-

<sup>1</sup> The points obtained from two experiments, which show extreme deviations, have been enclosed in parentheses. It is possible that the deviation of these points is the result of experimental error. In one of them poor analytical checks were obtained on one sample. Triplicate determinations were made. The second and third analyses agreed and gave the high value recorded. The first gave a much lower value. If the latter were used the point would fall in the same line as the others. The other value enclosed in parentheses was obtained in the course of a complicated experiment aimed at the simultaneous solution of another problem. It is possible that samples may have been mixed as the results were in other respects out of keeping with those obtained in similar experiments. The values have been retained because there was nothing but internal evidence to warrant their exclusion and the total number of observations is not sufficient to permit us to say that such variations are, in themselves, impossible.

# Peters, Bulger, and Eisenman

TABLE I

Oxygen capacity (1)	CO <sub>2</sub> content at 40 mm (2)	$\Delta[\text{CO}_2]_{10-30}$ blood (3)	$\Delta[\text{CO}_2]_{10-30}$ plasma (4)	$\frac{\Delta[\text{BHCO}_2]}{\Delta\text{pH}}$ blood (5)	$\frac{\Delta[\text{BHCO}_2]}{\Delta\text{pH}}$ plasma. (6)
<i>vols per cent</i>	<i>vols per cent</i>	<i>vols per cent</i>	<i>vols per cent</i>		
2 65	41 75	6 67		-18 2	
4 23	52 00	7 71		-22 2	
4 65	33 50	7 67		-25 1	
8 36	37 95	9 17		-32 1	
8 58	37 05	8 66		-29 1	
9 73	30 65	9 64		-40 9	
10 13	41 80	10 47		-40 0	
10 40	46 25	10 52		-38 5	
10 70	33 50	9 67		-38 6	
10 83	48 45	10 69		-39 4	
11 57	47 40	11 70		-46 2	
12 38	27 54	10 15	10 80	-49 8	-52 8
12 60	45 10	11 10		-43 7	
15 08	36 35	10 41		-43 0	
15 60	38 00	10 55		-44 1	
16 00	53 46	12 55		-49 6	
16 10	39 70	10 47	12 12	-41 2	-51 2
16 20	28 87	10 80	11 60	-55 6	-56 7
16 49	46 73	11 24	13 66	-43 7	-57 1
17 05	46 77	13 14	14 35	-56 8	-59 8
17 10	36 00	11 17		-49 2	
17 48	52 20	12 80	13 55	-52 0	-52 9
17 76	47 18	12 45	13 90	-52 4	-57 4
17 86	47 93	12 10	13 58	-49 0	-54 8
18 31	45 92	11 75	11 15	-47 9	-41 0
18 40	42 70	12 80		-56 9	
18 45	47 65	12 01		-49 3	
18 50	40 51	12 80		-62 0	
18 58	45 61	12 55	14 00	-53 8	-58 8
18 66	45 00	13 20		-58 9	-48 4
18 69	41 39	13 65	12 17	-67 1	
18 83	44 40	12 18		-52 1	
18 90	52 28	14 27	15 60	-62 8	-65 4
19 10	39 20	12 84		-61 8	
19 20	49 00	10 53*		-38 5*	
19 33	45 20	13 25		-60 2	
19 90	39 55	12 82		-61 3	
19 93	41 15	13 46		-65 5	
20 00	45 00	12 40	14 35	-53 2	-61 3
20 28	45 22	16 24*	18 31*	-89 2*	-95 8*

\*See foot-note 1

TABLE I—*Concluded.*

Oxygen capacity (1)	CO <sub>2</sub> content at 40 mm (2)	Δ[CO <sub>2</sub> ] <sub>60-30</sub> blood (3)	Δ[CO <sub>2</sub> ] <sub>60-30</sub> plasma (4)	$\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$ blood (5)	$\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$ plasma (6)
vols per cent	vols per cent	vols per cent	vols per cent		
20 98	33 35	13 01		-73 1	
21 30	43 12	13 65	15 33	-65 2	-70 6
21 30	53 00	14 15	15 70	-61 8	-64 5
21 31	44 48	13 57	15 32	-62 8	-69 1 .
21 33	46 40	14 44		-69 6	
21 40	39 70	13 40		-66 6	
21 60	39 10	12 46		-58 5	
21 75	56 76	13 45	14 25	-54 6	-54 2
22 40	44 38	13 57	16 30	-63 8	-78 8
23 60	53 96	14 38	17 26	-62 4	-75 7
28 70	38 18	15 42	17 63	-95 2	-97 4

more, there is no systematic difference between the results obtained from the two series of experiments. There is a definite tendency for the slopes of the curves to increase as the oxygen capacity increases. The lines AB (Chart 1, I and II) represent

CHART 1 I The relation of oxygen capacity to  $-\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  between 30 and 60 mm of CO<sub>2</sub> tension in both series of experiments (see Table V).

II The relation of oxygen capacity to Δ [CO<sub>2</sub>]<sub>60-30</sub> in both series of experiments (see Table V)

The two charts are drawn on different scales to render them comparable (For explanation of values in parentheses see foot-note 1 ) The lines AB indicate the average variations of  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  and Δ [CO<sub>2</sub>]<sub>60-30</sub> with changes of oxygen capacity and are defined by the equations:

$$-\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}} = 25.0 (\text{oxygen capacity}) + 10.5$$

and

$$\Delta[\text{CO}_2]_{60-30} = 0.334 (\text{O}_2 \text{ capacity}) + 6.3$$

The lines OC represent the calculated buffer values of the cells alone and are defined by the equations

$$-\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}} = 27.7 (\text{oxygen capacity})$$

and

$$\Delta[\text{CO}_2]_{60-30} = 0.470 (\text{oxygen capacity})$$

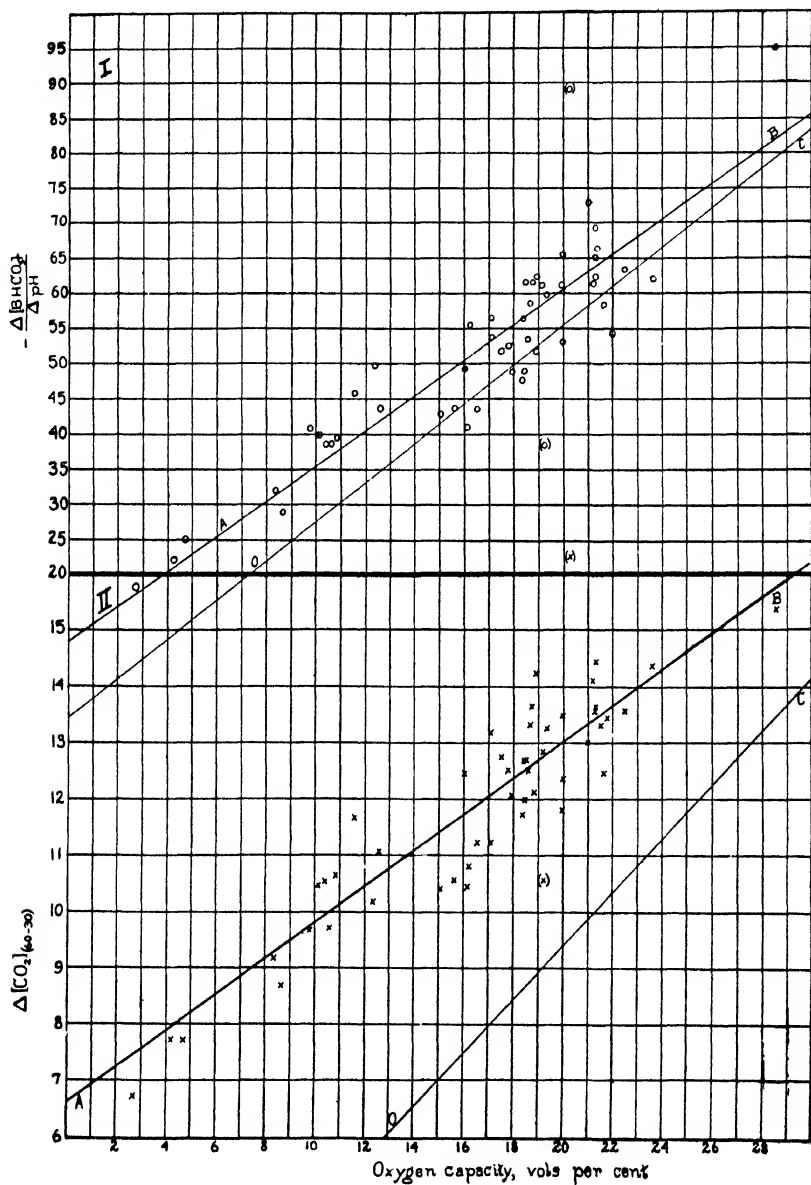


CHART 1



the best straight lines that can be constructed through the points on the two charts. The equations of these lines are:

$$-\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}} = 25.0h + 10.5 \quad (1)$$

and

$$\Delta[\text{CO}_2]_{40-30} = 0.334h + 6.3 \quad (2)$$

where  $h$  = oxygen capacity, expressed in volumes per cent. Although the points are not evenly distributed along the course of the curves, there is nothing in their scattering to indicate that the curves are other than straight lines.

Van Slyke (6) proposed the ratio  $\frac{\Delta[\text{B}]}{\Delta\text{pH}}$  as the best expression for the buffer value of a solution. With Hastings, Heidelberger, and Neill (8), he further showed that, in a solution of sodium hemoglobinate exposed to different CO<sub>2</sub> tensions, the value of the buffers other than bicarbonate (in this case the hemoglobinate alone) could be expressed by the ratio  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$ . This ratio apparently varied directly with the concentration of hemoglobin in these solutions. The molecular buffer value of horse hemoglobin was estimated by this method as 2.64.

In this study, Van Slyke and his coworkers were dealing with a homogeneous solution under comparatively constant conditions. The relation of [BHCO<sub>3</sub>] to pH, under these conditions, was investigated over a considerable range and was found to be rectilinear. Van Slyke, Hastings, and Neill (9) then employed the same ratio for the calculation of the value of buffers other than hemoglobin in horse blood, by difference. This involved three assumptions: (a) that the relation of  $\Delta[\text{BHCO}_3]$  to  $\Delta\text{pH}$  for whole blood was rectilinear; (b) that the buffer value of a mixed solution was a simple additive function of the values of the individual buffers in that solution; and (c) that the introduction into the system of a second phase and a membrane did not in itself affect the buffer value of any one of the constituents on either side of the membrane. Van Slyke, Hastings, and Neill presented the work only as a tentative approximation of the values of the buffers of whole blood. The subsequent studies of Van Slyke, Wu, and

McLean (10) have shown that the primary assumptions on which the approximations were based were not exact. Van Slyke, Wu, and McLean (10) have shown that the  $[\text{BHCO}_3]$ , pH curve does show a slight but definite curvature convex to the pH axis, thus explaining and supporting the superiority of our logarithmic relation as a means of defining the carbon dioxide absorption curve. Under these circumstances the buffer value, measured by Van Slyke's method, varies in the same blood with the carbon dioxide tension and the bicarbonate content. Furthermore, these variations become greater as the slope increases. This may explain the fact that at higher oxygen capacities the degree of scattering in Chart 1 increases. That the relation of  $\Delta[\text{BHCO}_3]$  to  $\Delta\text{pH}$  is not rectilinear in whole blood follows inevitably from the conclusions of the preceding paper of this series (7). The absorption curve of whole blood is more nearly defined by the equation  $\Delta \log [\text{CO}_2] / \Delta \log [\text{pCO}_2] = \text{a constant}$ . If this is the case it can be shown that the absorption curve drawn as  $y = [\text{BHCO}_3]$ ,  $x = \text{pH}$  is a curve convex to the abscissa and that the degree of curvature increases as the slope of the curve becomes greater.

Although the ratio  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  is not constant in a given blood under different conditions, nevertheless, under the restricted conditions of the experiments of Van Slyke, Hastings, and Neill, and those here presented the buffer value of a series of bloods measured by this ratio may be comparable and may prove to be additive. In this case the line AB in Chart 1, I, must represent the variations in the buffer value of blood due to changes in cell volume, and the point where AB intersects the  $y$  axis,  $y = 10.5$ , should represent the buffer value of "separated" plasma. The latter value, according to the data obtained from a few experiments, is of the right order of magnitude. If the buffer values in blood are additive when the blood contains 50 per cent cells the average plasma buffer value should be  $0.5 \times 10.5$  or 5.25. According to data obtained in this laboratory the average ratio, (*oxygen capacity*). (*cell volume*), in human blood is 0.464. Therefore, at an oxygen capacity of 23.2 volumes per cent the blood should contain equal amounts of cells and plasma. At 23.2 volumes per cent

O<sub>2</sub> the line AB lies at  $-\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}} = 69.5$ . The buffer value of the cells, therefore, should be  $69.5 - 5.25 = 64.25$ . The line OC, drawn through this point from the origin and defined by the equation,

$$\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}} = 2.77h \quad (3)$$

should represent the buffer value of the cells. As both slope and oxygen capacity are calculated directly from gas volume, the ratios are equivalent to molecular ratios and one can say that the average molecular buffer value of the blood cells in these experiments is 2.77. This value is quite comparable to the value 2.64 obtained from pure horse hemoglobin by Van Slyke, Hastings, Heidelberger, and Neill (8). If the other cell buffers are of little importance the observed difference is quite possible.

Although from a study of average values and the character of the line AB the findings in both experiments are reasonably in keeping with the hypothesis of additive buffer values, a study of the individual experiments is not so satisfactory. In several instances the total buffer value of blood is less than the average buffer value of the cells alone. Of course, it may be that the non-hemoglobin cell buffer values in these experiments are lower. In that case the buffer value of hemoglobin, which has been employed as a measure of cell volume,<sup>2</sup> must be correspondingly reduced. The value for the buffer value of human hemoglobin is hardly likely to prove considerably lower than that of horse hemoglobin. However, unless it were considerably lower and unless the non-hemoglobin intracellular buffers were insignificant the extracellular buffers in some of our experiments would have to be smaller than any available experimental data would lead us to believe. Experimental errors may be responsible for the variability of the observed values and especially for the low values above mentioned. Aside from the fact that the control work previously cited (No. I of this series (5)) gives no evidence of errors sufficiently large to explain the variations shown in these

<sup>2</sup> If the chart is drawn with  $x$  = cell volume there is no significant change in the distribution of the points or the character of the curves.

experiments,<sup>3</sup> there is collateral evidence that experimental errors alone are not responsible.

Van Slyke, Hastings, and Neill (9) determined the buffer value of horse blood and compared it with that of pure horse hemoglobin. The difference they ascribed to the other buffers of the blood. From two experiments on separated horse plasma we have obtained buffer values of 11.33 and 13.64, with an average of 12.49. The ratio (*oxygen capacity*) : (*cell volume*) in these experiments was 0.48. These values are substantially similar to those calculated from the work of other observers. From these values and the molecular buffer value of horse hemoglobin, 2.64, Chart 2 has been constructed. In this chart OC represents the buffer value of horse hemoglobin and AB the buffer value of horse hemoglobin plus that of horse plasma. If it be assumed that hemoglobin is the only important buffer in the cells, AB may be considered as representing the buffer value of whole horse blood with varying cell volume and is comparable to the line AB in Chart 1, I. The dots represent the buffer values of horse blood observed by Van Slyke, Hastings, and Neill. It is at once apparent that the values of the buffers of blood other than hemoglobin are altogether too small. The mixing together of cells and plasma appears to have reduced the buffer value of either cells or plasma or both. Van Slyke, Wu, and McLean (10) found that the introduction into the system of a second phase and a cell membrane lowered the buffer value of the cell contents estimated as  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$ . They pointed out that the explanation of this effect lay in the fact that the  $\Delta\text{pH}$  employed as the denominator of the ratio was that of the serum and not that of the cell contents, which was smaller; *i.e.*,  $\Delta\text{pH}_c < \Delta\text{pH}_s$ . In this connection our own findings are only confirmatory of theirs. The absolute values of the cell buffers cannot be correctly estimated from the values of  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}_s}$ . If, however, the apparent diminution of

\* If the  $\text{CO}_2$  determinations were assumed to be in error by twice the mean error, or  $2 \times 0.2$  vols per cent and these errors were additive, the change of the ratio —  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  would be about 1.0. The deviations here observed are, however, of an entirely different order of magnitude.

the effect of cell buffers that results from the interposition of the cell membrane were a direct function of the cell volume or hemoglobin alone,  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}_s}$  should still bear a definite relation to oxygen capacity or cell volume, even if this relation were not

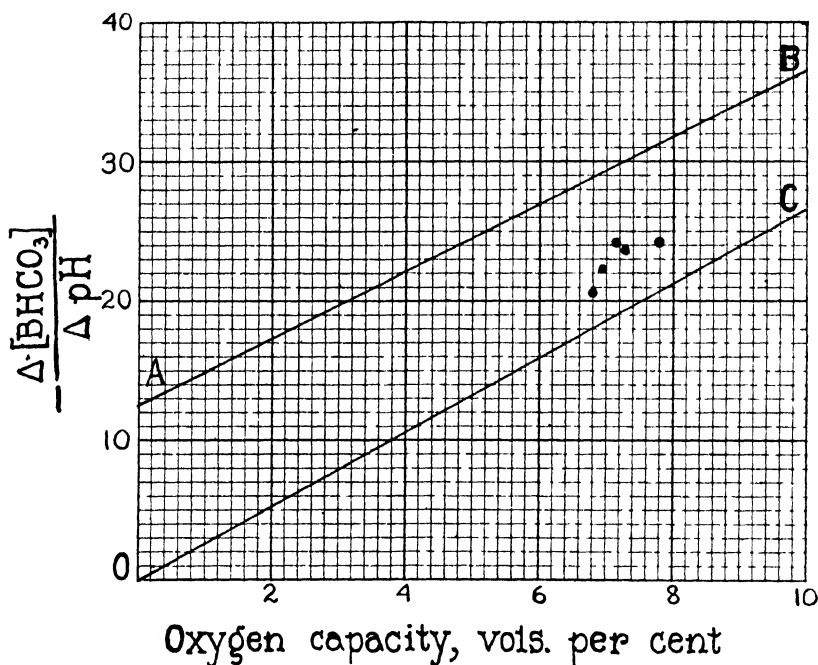


CHART 2 Abscissa = oxygen capacity in vols per cent, ordinate =  $-\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$ .

. = observations of Van Slyke, Hastings, and Neill on horse blood

Line OC = buffer value of horse hemoglobin according to Van Slyke, Hastings, Heidelberger, and Neill

Line AB = calculated buffer value of horse hemoglobin + horse plasma

linear. The extreme scattering in Chart 1, however, suggests that other factors of considerable importance enter into the determination of the slope.

It seemed possible that this scattering might prove less if comparisons were made at constant pH rather than at constant CO<sub>2</sub> tension. Calcula-

tions made to test this point proved that isohydronic comparison did not diminish scattering

To study the effect of altering cell volume on the buffer value and the CO<sub>2</sub> absorption curve more exactly the following experiments were performed.

*Experiment 1* —75 cc of blood from the arm of patient No 6657, prevented from coagulating by the addition of 0.2 gm of potassium oxalate, were saturated with 40 mm of CO<sub>2</sub> in air at 38°C. Two 8 cc portions of the blood were saturated again at 30 and 60 mm. The remainder was centrifuged in large tubes without contact with air. Two 8 cc portions of plasma were

TABLE II  
*Experiment 1.*

No	Sample	O <sub>2</sub> capac- ity	CO <sub>2</sub> content at		$\Delta[\text{CO}_2]_{\text{Hb-30}}$	Cell volume at 30 mm	$\frac{\Delta[\text{BHCO}_2]}{\Delta\text{pH}}$
			30 mm	60 mm			
		vols per cent	vols per cent	vols per cent	vols per cent	vols per cent	
1	"Separated" plasma	0	48 08	54 30	6 22	0	-15 2
2	"Diluted" blood.	8 74	41 89	50 77	8 88	18 45	-28 5
3	Whole blood	18 50	34 56	47 60	13 04	39 40	-49 3
4	"Concentrated" blood	26 35	31 50	43 32	11 82	56 45	-56 0

removed and saturated at 30 and 60 mm. Unequal mixtures of plasma and cells were then produced without loss of CO<sub>2</sub> so as to imitate an anemia and polycythemia. Samples of each of these mixtures were then saturated at 30 and 60 mm.

Analyses of the various samples give results shown in Table II.

From the results of the experiment it is at once apparent that the slopes of the curves, however estimated, increase in the first three specimens while the levels of the curves steadily diminish. The "concentrated" blood proved difficult to handle because of its viscosity and because the cells showed a tendency to clump so that it was impossible to obtain a good mixture. The results from this specimen should therefore be discarded. Although the buffer values  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  increase with the hemoglobin the relation of buffer value to hemoglobin is not linear even in the first three

samples.<sup>4</sup> If the values are plotted as they are in Chart 3, I, *a*, as  $x = -\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$ ,  $y = (\text{oxygen capacity})$  and a line be drawn connecting the "separated" plasma and whole blood points it will give at  $(\text{oxygen capacity}) = 8.74$  a value of 31.1, instead of the observed 28.5 for  $-\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$ .

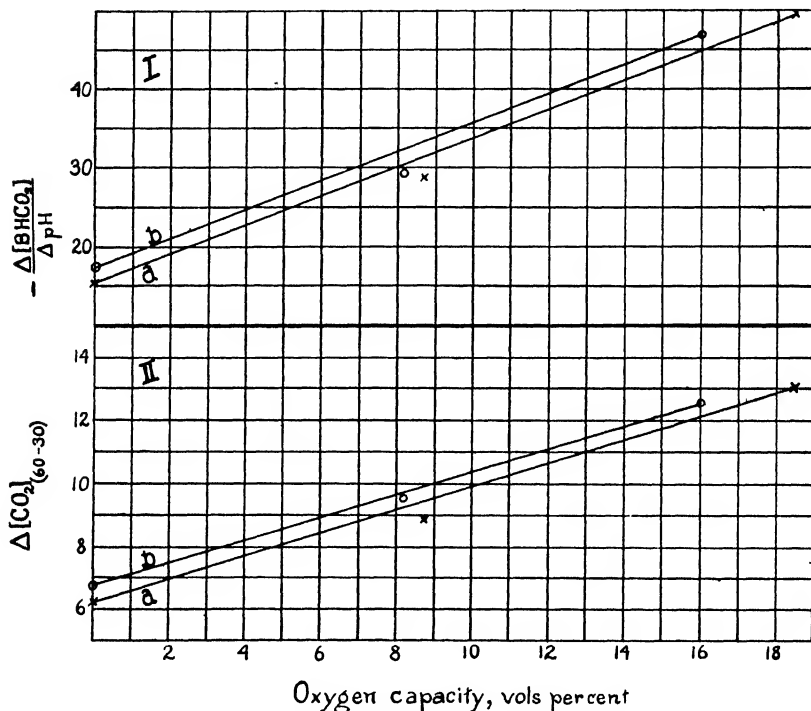


CHART 3. Results of Experiments 1 and 2. Abscissa = oxygen capacity in vols. per cent; ordinate in No. I =  $-\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$ ; ordinate in No. II =  $\Delta[\text{CO}_2]_{60-30}$ .

x—o = Experiment 1.

o—b = Experiment 2.

<sup>4</sup> In calculating the slopes of the different specimens a constant  $\text{pK}_1$  of 6.1 was assumed for all points. This is, of course, improper, but substitution of corrected  $\text{pK}_1$  values does not alter the results.

The experiment, of course, demanded so much manipulation that there was a possibility that considerable errors might enter if the greatest care was not exercised. The data can be submitted to a certain amount of analysis. If, as we believe, the transfers of cells and plasma were effected without loss of  $\text{CO}_2$ , at 40 mm. the concentration of  $\text{CO}_2$  in the plasma and in the cells of all specimens should be the same. If all the curves are plotted according to the straight line logarithmic relation, the  $\text{CO}_2$  content at 40 mm. may be calculated. Within the narrow limits of 30 and 60 mm. of  $\text{CO}_2$  the result should not be far from the actual 40 mm. value. The values obtained by this method for the  $\text{CO}_2$  content at 40 mm. are: plasma, 50.8 volumes per cent; low cells, 45.5 volumes per cent; whole blood, 39.5 volumes per cent; and high cells, 36.0 volumes per cent. As the differences between the various specimens are due to the changes in cell volume they should be proportional to the hemoglobin content, or

$$\frac{(50.6 - 45.4)}{8.74} = \frac{(50.6 - 39.5)}{18.5} = \frac{(50.6 - 35.9)}{26.35}$$

should be true.

$$\frac{(50.6 - 45.4)}{8.74} = 0.598, \quad \frac{(50.6 - 39.5)}{18.5} = 0.600, \text{ and } \frac{(50.6 - 35.9)}{26.35} = 0.556$$

It is evident that the agreement is satisfactory as far as the "diluted" blood and the whole blood are concerned, but that the results on "concentrated" blood cannot be accepted.

If the results on the first three specimens, "separated" plasma, "diluted" blood, and whole blood, are to be accepted it is again evident that buffer values cannot be considered as additive in whole blood. In this case, a change in cell volume alone gives a curve which is convex to the abscissa when  $x = O_2 - \text{capacity}$  and  $y = \frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$

*Experiment 2*—About 75 cc. of blood, from the arm vein of patient No. 22684, prevented from coagulation by the addition of potassium oxalate, were saturated with 30 mm. of  $\text{CO}_2$  in air at  $38^\circ\text{C}$ . On a sample of this blood the following determinations were made:  $\text{CO}_2$  content, oxygen capacity, and hematocrit value. Another portion was at once centrifuged. A sam-



ple of the plasma was removed and analyzed for CO<sub>2</sub>. Another sample of the plasma was saturated with 60 mm of CO<sub>2</sub> in air at 38°C. and analyzed for CO<sub>2</sub> to give the absorption curve of the "separated" plasma. A third portion of the plasma was mixed with an equal amount of the whole blood which had been saturated at 30 mm and the mixture was saturated with 60 mm. of CO<sub>2</sub> in air at 38°C. (This we shall call "diluted" blood.) A sample of this blood was analyzed for CO<sub>2</sub> content, oxygen capacity, and hematocrit value. The remainder was centrifuged and its plasma analyzed for CO<sub>2</sub>. A portion of the original blood was saturated at 60 mm and a sample analyzed for CO<sub>2</sub>, oxygen capacity, and hematocrit value. Plasma was also obtained from this blood and analyzed for CO<sub>2</sub>. In this way absorption curves between 30 and 60 mm of CO<sub>2</sub> tension were obtained for. "separated" plasma, "diluted" blood and its "true" plasma, and whole

TABLE III  
*Experiment 2*

Sample	CO <sub>2</sub> content at		Difference $\Delta[\text{CO}_2]_{0-30}$	O <sub>2</sub> capacity at		Cell volume (hematocrit) at		$\frac{\Delta[\text{HCO}_3]}{\Delta\text{pH}}$
	30 mm	60 mm		30 mm	60 mm	30 mm	60 mm	
	<i>vols per cent</i>	<i>vols per cent</i>	<i>vols per cent</i>	<i>vols per cent</i>	<i>vols per cent</i>	<i>vols per cent</i>	<i>vols per cent</i>	
"Separated" plasma	56 65	63 40	6 75					-17 4
"Diluted" blood	(52 55)	62 05	9 50	(8 19)	8 19	16 00	16 65	-29 6
Whole blood	48 60	61 15	12 55	15 98	15 98	31 70	33 50	-47 1
"True" plasma of "di- luted" blood	(56 65)	65 20	8 55					-25 4
"True" plasma of whole blood	(56 65)	68 55	11 90					-43 4

blood and its "true" plasma, with corresponding oxygen capacities and hematocrit values. The reader will note that the "diluted" blood at 30 mm was not analyzed for CO<sub>2</sub>. It was assumed that all transfers were made without loss of CO<sub>2</sub> and that adequate admixture of cells and plasma was maintained throughout the experiment. If these assumptions were correct the CO<sub>2</sub> content of the diluted blood at 30 mm could be calculated from the values obtained from the CO<sub>2</sub> content, oxygen capacity, and hematocrit values of whole blood at 30 mm, the CO<sub>2</sub> content of the plasma at 30 mm, and the oxygen capacity of the diluted blood at 60 mm. The economy of time and material effected by the elimination of one blood analysis and one plasma analysis must be apparent at once when the complicated nature of the experimental procedure and the number of manipulations entailed are taken into consideration. One means of checking the accuracy of the technique was employed. A hematocrit determination was made on the "diluted" blood at 30 mm. The results of the experiment are given in Table III, calculated values being enclosed in parentheses.

As far as Experiment 2 is comparable with Experiment 1 the results are identical. Again, as the oxygen capacity of the blood increases the slope increases while the level of the curve falls.

However, the change in slope, measured by the ratio,  $-\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$ ,

is not proportional to the change in oxygen capacity. The degree of deviation from the direct relation is shown in Chart 3, I, *b*, and is about the same as that found in Experiment 1. The line connecting the "separated" plasma and whole blood points at an oxygen capacity of 8.19 volumes per cent gives a value of 32.62 for  $-\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$ , instead of the observed 29.60.

If, in these experiments, the simple arithmetical difference between the  $\text{CO}_2$  contents at 30 and 60 mm.,  $\Delta[\text{CO}_2]_{60-30}$ , is used as a measure of the slope and is plotted directly against the oxygen capacity, as has been done in Chart 3, II, *a* and *b*, the three points obtained from each experiment lie in a straight line. So exact is this relation that the line connecting the "separated" plasma and whole blood points is only 0.6 volume per cent above the observed value obtained from the "diluted" blood in the first experiment and only 0.2 volume per cent away in the second experiment. The explanation of this agreement lies in the fact that all the specimens in each experiment agree at one point as regards the concentration of  $\text{CO}_2$  in cells and in the plasma. The divergences in height and slope are entirely due to the presence of varying amounts of cells and the redistribution of electrolytes that results from this variation. We are dealing with conditions in which, as far as slope is concerned, only one variable changes. These are the conditions under which one would be most likely to obtain a linear relation between the slope and that variable which is, in this case cell volume, or its direct function, hemoglobin. So close an agreement as this is hardly likely to be merely fortuitous and may be considered as contributory evidence that experimental errors have been avoided.<sup>5</sup>

<sup>5</sup> It seems quite proper, to point out that such results are in themselves a certain guarantee of technical accuracy. Where conclusions are so largely dependent on the accuracy of analytical data every point that bears on this aspect of the work is worthy of emphasis. Even with the most perfect technique and far simpler procedures than those involved in these experi-

Although in these experiments the relation of  $\Delta[\text{CO}_2]_{60-30}$  to the oxygen capacity is linear, this does not hold except in a general way for the series of experiments on different bloods studied between 30 and 60 mm. because other variables enter into these experiments to obscure the relation. Chart 1 shows that the scattering is about the same when either  $-\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  or  $\Delta[\text{CO}_2]_{60-30}$  is plotted against oxygen capacity.

Barr, Himwich, and Green, and Barr and Himwich (11) state that, as the CO<sub>2</sub> absorption curve falls (*i.e.* as the available base diminishes), the slope of the curve decreases. In their experiments the diminution of available base was effected in the body by means of exercise. There was undoubtedly some alteration in oxygen capacity associated with the change of bicarbonate. Although the authors recognized that such a change of oxygen capacity occurred, they failed to determine the extent of the change. It was probably not considerable and can be neglected without introducing a significant error. In comparing the slopes of their absorption curves they did not use the Van Slyke ratio,  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$ , nor any other standard which permitted accurate comparison. We have calculated the CO<sub>2</sub> content at 30, 60, and 40 mm. for all suitable curves in Barr and Himwich's series by the use of our straight line logarithmic equation (7). (See Table IV.) Although, at first glance the results do suggest that there is a direct relationship between the height and the slope of the absorption curve, on careful analysis the association seems to be merely accidental. In the table the values obtained from each subject are arranged in order of descending magnitude of available base. In every case there is a sharp drop in the value of the slope, whether measured as  $\Delta[\text{CO}_2]_{60-30}$  or  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$ , with the first

ments, however, 100 per cent success cannot be expected. A third experiment, the second in chronological order, was a complete failure. It is omitted not because it proved incompatible with the other experiments; but because it gave indubitable evidences of technical errors in the treatment of one sample of "diluted" blood. It would add nothing to include it in the paper. It is fair to state that no relation could be found in this experiment between either  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  or  $\Delta[\text{CO}_2]_{60-30}$  and the oxygen capacity.

diminution of  $[\text{CO}_2]$ . Beyond this, with further reduction of  $[\text{CO}_2]$ , the slope remains constant or varies without relation to the

TABLE IV.

Subject	Date	Experiment	CO <sub>2</sub> content of blood at 40 mm.	$\Delta[\text{CO}_2]_{\text{pH}}^{\text{pH}}$	$\frac{\Delta[\text{HCO}_3]}{\Delta\text{pH}}$
			vols per cent	vols per cent	
D. P. B.	Oct. 17	Venous blood before exercise.	46 6	15 0	-74 2
	Dec 13	" " after "	37 3	11 9	-55 0
	June 20	" " " "	34 6	13 2	-73 2
	Dec 23	" " " "	32 0	11 1	-53 8
	Mar 31	" " " "	30 4	11 6	-62 2
	Apr 17	Arterial " " "	29 5	10 9	-55 4
	" 10	Venous " " "	27 6	11 0	-59 6
H. E. H.	" 14	Arterial " before "	50 1	14 3	-64 6
	Mar 22	" " " "	48 7	12 8	-53.5
	Apr. 21	" " after "	45 2	10 4	-52 9
	" 14	" " before "	41 0	10 6	-57 9
	Mar 29	" " after "	40 9	12 0	-52 4
	Apr 21	" " " "	36 9	12 6	-62 4
	" 5	" " " "	32 4	11 2	-54 2
N. P. L.	Nov 18	Venous " before "	47 6	16 1	-83 8
	Apr. 24	" " after "	36 7	13 5	-72 8
	Nov 18	" " " "	34 6	12 7	-67 0
	Apr. 24	Arterial " " "	33 0	13 2	-77 0
J. McL.	Nov. 30	" " before "	48 4	12 0	-48 3
	" 30	" " after "	32 5	7 5	-24 9
K. G. H.		Venous " before "	48 5	14 9	-70 8
		" " after "	30 6	11 8	-64 6
M. L.	Apr. 7	Arterial " before "	46 2	14 9	-73.6
	" 19	Venous " after "	33 7	11 6	-56 5
	" 7	Arterial " " "	31 6	11 0	-53 2
	" 19	" " " "	30 3	11 6	-62 2
H. B. R.	" 12	" " before "	46 7	15 2	-75 5
	" 27	Venous " after "	42 6	12 4	-54 8
	" 12	" " " "	40 1	12 3	-56 0
	" 27	Arterial " " "	37 9	12 6	-61 3

height of the absorption curve. In every instance the first fall in the height of the curve was produced by exercise. Subsequent reductions are dependent on the degree of muscular exertion only.

Barr and Himwich have shown that exercise produces profound alterations in the nature of the blood, of which the lowered alkaline reserve is only one indication. Their results can be interpreted to prove that some factor evoked by exercise alters the slope of the absorption curve. Our own results (see Table I) show no evidence of a relation between the height and the slope of the absorption curve.

Thus far no mention has been made of the plasma figures. In most cases the slope of the "true" plasma curve is greater than that of the whole blood. This is in keeping with the results of the majority of observers. In a few instances this relation is reversed and the whole blood curve has the greater slope. In general, the slope of the curve of the "true" plasma varies with that of the blood from which it was derived; but the relation is not exact. The number of experiments available for comparison of the two is too small to permit any accurate deductions; but apparently the correlation between oxygen capacity and the slope of the plasma curve is not so good as that obtained with whole blood. In Experiment 2, no linear relation was found between the slope of the "true" plasma and the oxygen capacity when either  $\Delta[\text{CO}_2]_{60-30}$  or  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  was employed as a measure of the slope.

From this experiment alone it is unsafe, however, to draw any conclusions.

The slope of "separated" plasma was determined on three different samples of blood under entirely comparable conditions. The variations observed were surprisingly large

$$\Delta[\text{CO}_2]_{60-30} = 4.10 \text{ to } 6.75, \frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}} = -7.1 \text{ to } -17.4$$

It is possible that this may represent one of the other important factors that determine the slope of whole blood. Two of these experiments are those described above; in the third the whole blood curve alone is available for comparison with the "separated" plasma curve. In all, hematocrit determinations were made so that it is possible to calculate what proportion of the slope is due to plasma alone and what proportion is due to the presence of the cells, in the following manner.

If  $m_b$  is the slope of blood and  $m_p$  the slope of "separated" plasma calculated either as  $\Delta [\text{CO}_2]_{60-30}$  or as  $-\frac{\Delta [\text{BHCO}_3]}{\Delta \text{pH}}$ , then

$$m_b - m_p (1 - hk) = m_c$$

where  $hk$  represents the cell volume and  $m_c$  the proportion of the slope due to the presence of the cells

The values thus obtained are shown in Table V and are plotted against corresponding oxygen capacity values in Chart 4. For comparison the uncorrected values are also shown in the chart.

TABLE V

Experiment No	Nature of specimen	Oxygen capacity	$\Delta [\text{CO}_2]_{60-30}$			$\frac{\Delta [\text{BHCO}_3]}{\Delta \text{pH}}$		
			Total	Plasma	Cell	Total	Plasma	Cell
		vols per cent	vols per cent	vols per cent	vols per cent			
1	"Separated" plasma	0	6 12	6 12	0	-15 2	-15 2	0
	"Diluted" blood	8 74	8 7	4 0	3 7	-28 5	-12 4	-16 1
	Whole blood	18 45	12 8	3 7	9 1	-49 3	-9 2	-40 1
2	"Separated" plasma	0	6 75	6 75	0	-17 5	-17 5	0
	"Diluted" blood	8 19	9 5	5 7	3 85	-30 7	-14 7	-16 0
	Whole blood	15 98	12 55	4 6	7 95	-49 6	-11 9	-37 7
3	"Separated" plasma	0	4 10	4 10	0	-7 1	-7 1	0
	Whole blood	15 60	10 6	3 4	7 2	-44 1	-4 8	-39 3

The correction certainly tends to bring the points into closer alignment when  $\Delta [\text{CO}_2]_{60-30}$  is used as the criterion of slope, but has little effect on the scattering of the  $-\frac{\Delta [\text{BHCO}_3]}{\Delta \text{pH}}$  values.

Finally, the cell buffer values shown in Chart 4, II, are quite different from those indicated by the line OC in Chart 1, I; while the OC lines in Charts 1, II and 4, I, are practically identical.

The assumption that values of individual buffers occurring in a complex system such as blood, when measured by the ratio  $\frac{\Delta [\text{BHCO}_3]}{\Delta \text{pH}}$ , can be considered as simple additive functions then,

appears to be unwarranted. Apparently the ratio cannot even be used with any accuracy as a measure of the *relative* effects of cell and plasma buffers in blood when the blood is subjected to uniform treatment that tends to limit variations.

Under the highly restricted conditions of these experiments the simple arithmetical difference  $\Delta[\text{CO}_2]_{60-30}$  is a satisfactory and practical measure of buffer. The experimental material available

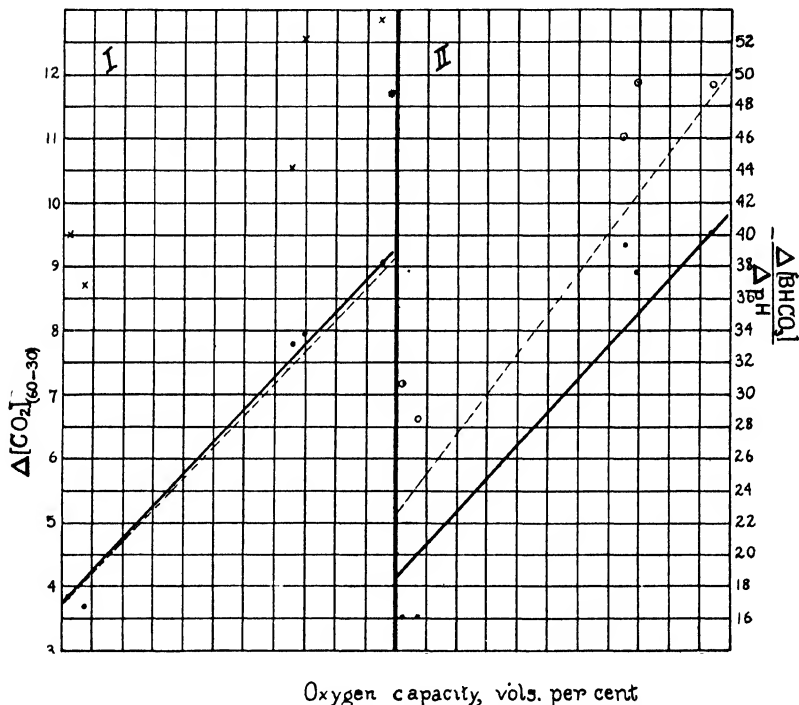


CHART 4 I Shows the relation of  $\Delta[\text{CO}_2]_{60-30}$  to oxygen capacity in three experiments in which values for "separated" plasma are also available (see Table V)

x = values obtained directly from blood samples.

. = the same values corrected for plasma and, therefore, may be taken to represent  $\Delta[\text{CO}_2]_{60-30}$  for cells alone.

The solid diagonal line is drawn from the origin to the highest observed point. The broken diagonal is the OC line transposed from Chart 1.

II. Shows the relation of  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  to oxygen capacity in the same experiments. The two charts are comparable.

for the estimation of the relative effects of cells and plasma, presented in the last chart, is too scanty to warrant drawing any conclusions. The results suggest, however, that the buffer value of "separated" plasma is more variable than it has been generally supposed, while the cell buffers are comparatively constant and vary almost entirely with the hemoglobin. Furthermore, it seems possible that the buffer values of individual constituents, or at least the two main phases, of the complex system, blood, when estimated as  $\Delta[\text{CO}_2]_{60-30}$  may be considered as simple additive functions.

It may be well to analyze this differential,  $\Delta[\text{CO}_2]_{60-30}$ , somewhat further. There is no reason for believing that there is any especial significance attached to the figures 60-30, it is probable, but not certain, that similar relations would obtain among a series of bloods compared between two other tensions, provided that the difference between the two tensions were sufficiently small. This does not imply that the general differential  $\frac{\Delta[\text{CO}_2]}{\Delta p\text{CO}_2}$  can be substituted for  $\Delta[\text{CO}_2]_{60-30}$ . The latter is a restricted form of the  $\frac{\Delta[\text{CO}_2]}{\Delta p\text{CO}_2}$  generalization in that the denominator remains constant.

Obviously the buffer value, measured by the ratio  $\frac{\Delta[\text{CO}_2]}{\Delta p\text{CO}_2}$ , would vary with the alkali reserve. From a theoretical point of view, perhaps  $\Delta[\text{BHCO}_3]$  is preferable to  $\Delta[\text{CO}_2]$ . At constant  $\text{CO}_2$  tension, however, the two are linear functions of one another. Under these circumstances nothing is to be gained by using  $[\text{BHCO}_3]$ , especially when the values of the solubility coefficients in whole blood are so uncertain.

Parsons and Poulton (12) following Barcroft, Bock, Hill, Parsons, Parsons, and Shoji (13) have considered that buffer value should be expressed as a change in  $C_H$  and have defined it as "the percentage volume of  $\text{CO}_2$  it is necessary to add to blood within the physiological range, to increase the  $C_H$  by  $1 \times 10^{-8}$ ".

Under these circumstances they find that increasing the fixed acid of the blood diminishes its buffer value. In proposing the relation of  $C_H$  to  $[\text{CO}_2]$  as a criterion of buffer value, Barcroft and his coworkers assumed that  $C_H$  and  $[\text{CO}_2]$  were linear functions. As we have pointed out (7) this assumption is greatly at variance with the facts.

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# STUDIES OF THE CARBON DIOXIDE ABSORPTION CURVE OF HUMAN BLOOD.

## V. THE CONSTRUCTION OF THE CO<sub>2</sub> ABSORPTION CURVE FROM ONE OBSERVED POINT.

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The prime object of the work presented in the preceding paper was not to test the validity of any hypotheses that dealt with the slope of the CO<sub>2</sub> absorption curve of blood, but to find a means of defining the absorption curve from one observed point. For this purpose it was necessary to study the factors that determined the slope of the curve. One of the most important of these is the hemoglobin concentration. The value of the intrinsic buffers of the plasma may be another. The work of Barr and Himwich (1)<sup>1</sup> suggests still other extraneous factors that may be active in the body. To date the result of the investigation has not been entirely satisfactory from the practical point of view because the nature of the determinants other than hemoglobin has not been ascertained. Nevertheless, these data permit a closer approximation of the slope of the curve than any previously available because they offer a correction for the effect of hemoglobin, at least.

In addition to the theoretical considerations already advanced, practical reasons have led us to choose as a measure of buffer values the simple arithmetical difference between the values of blood CO<sub>2</sub> content at 60 and 30 mm. of CO<sub>2</sub> tension (which we have designated  $\Delta[\text{CO}_2]_{60-30}$ ) rather than the ratio  $\frac{\Delta[\text{BHCO}_3]}{\Delta\text{pH}}$  recommended by Van Slyke (2). In the first place the correlation

<sup>1</sup> See the "Discussion" in the preceding paper.

of the difference with oxygen capacity in our experiments is quite as good as that of the ratio. In the second place the derivation

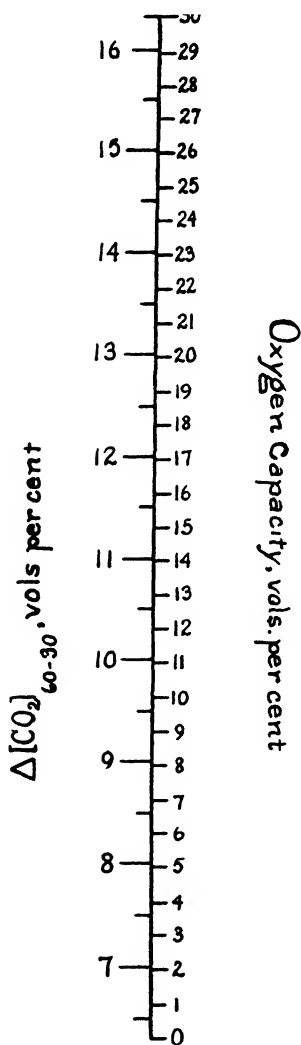


CHART 1.

demands the introduction of no mathematical formulas involving solubility coefficients or  $pK_A$  values. Finally, it is more easily

combined with the logarithmic formula suggested in Paper III of this series (3). The average relation of  $\Delta[\text{CO}_2]_{60-30}$  to oxygen capacity, according to our experimental data, is defined by the equation

$$\Delta[\text{CO}_2]_{60-30} = 0.334 (\text{oxygen capacity}) + 6.3$$

By means of Chart 1 or this equation  $\Delta[\text{CO}_2]_{60-30}$  can be determined from the oxygen capacity. If the  $\text{CO}_2$  content of the blood at any known tension is plotted on logarithmic paper, on which the abscissa represents  $\text{CO}_2$  tension and the ordinate  $\text{CO}_2$  content, a line drawn through this point to subtend, between the 30 and 60 mm.  $\text{CO}_2$  tension lines, the volumes per cent of  $\text{CO}_2$  corresponding to the oxygen capacity (derived by the above equation) will represent the absorption curve. The average deviation of  $\Delta[\text{CO}_2]_{60-30}$  from the mean in these experiments is 0.5 volume per cent. If it is more convenient, cell volume can be substituted for oxygen capacity by means of the equation

$$(\text{Oxygen capacity}) = 0.464 (\text{cell volume})$$

where both oxygen capacity and cell volume are expressed in terms of volumes per cent.

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# STUDIES OF THE CARBON DIOXIDE ABSORPTION CURVE OF HUMAN BLOOD.

## VI. THE RELATIONSHIP OF THE CO<sub>2</sub> OF BLOOD TO THAT OF PLASMA.

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In the first paper of this series (1) was proposed a set of empirical corrections for the value of the constant  $pK_1$  of the Henderson-Hasselbalch equation ( $pH = pK_1 + \log \frac{[BHCO_3]}{[H_2CO_3]}$ ) when the latter was employed for the calculation of the pH of whole blood.

Since then considerable additional experiments have been performed which offer suitable material for further evaluation of these corrections. In the light of these observations and in view of the fact that Parsons and Poulton (2), Van Slyke, Wu, and McLean (3), and Cullen and Robinson (4) have subsequently discussed the subject it seems well to reconsider our original conclusions.

### EXPERIMENTAL.

The oxygen capacity, cell volume, and CO<sub>2</sub> content of whole blood and the CO<sub>2</sub> content of the "true" plasma at 40 mm. of CO<sub>2</sub> tension have been determined on 87 specimens of blood. The same analyses have been made on 22 specimens of blood at 30 and 60 mm. of CO<sub>2</sub> tension. It is possible by means of the logarithmic relation presented in a previous paper (5) to reduce the latter series also to 40 mm.<sup>1</sup> The results of these experiments are given

<sup>1</sup> In the calculation of the cell volume at 40 mm. it was assumed that the exchange of water between cells and plasma was proportionate to the change of CO<sub>2</sub>. This may involve a certain error, but the total change in hematocrit values between 30 and 60 is so small that the error is negligible.

in Table III. The technique employed has been described in the first paper of this series (1).

#### DISCUSSION.

Reduced to its simplest terms the problem resolves itself into an attempt to predict the bicarbonate content of "true" plasma from the bicarbonate content of the blood from which it was derived (1). The difference between these two is determined chiefly by the concentrations of hemoglobin and of bicarbonate in the blood (1 to 4, and 6). Joffe and Poulton (7) were the first to make any extensive study of the relation of the CO<sub>2</sub> absorption curve of whole blood to that of its plasma. Their studies were confined to a few normal bloods in which the only important variable was carbon dioxide tension. Campbell, Hunt, and Poulton (8) found that, if the carbon dioxide content of plasma within physiological limits of CO<sub>2</sub> tension was plotted on rectangular coordinate paper against the carbon dioxide content of the corresponding blood, the result was a diagonal line. The height of this line varied with the oxygen capacity. Such a straight line relationship was to be expected in view of the fact that Joffe and Poulton had already shown that the absorption curve of the "true" plasma closely paralleled that of the blood. The graphic chart recommended by Campbell, Hunt, and Poulton for the prediction of plasma CO<sub>2</sub> content from that of blood does not make any allowance for those factors that cause variations from the average, and is therefore inaccurate. This inaccuracy has been demonstrated by Parsons and Poulton (2). Warburg (6) developed the Henderson-Hasselbalch equation, adding to it factors that corrected for the effect of hemoglobin and bicarbonate concentrations. These corrections involved the definition of the relation of hemoglobin concentration to cell volume and of the variations in the distribution of bicarbonate between cells and plasma. Our own data, which dealt with variations in the factors, differed from those of Warburg and it was this that led us to discard his correction values and substitute others. In one respect we followed his lead in that the correction proposed ( $\Delta pK_1$ ) was applied directly to the Henderson-Hasselbalch equation, which was altered to the form,

$$\text{pH} = \text{pK}_1 + \log \frac{[\text{BHCO}_2]}{[\text{H}_2\text{CO}_2]} + \Delta \text{pK}_1$$

Cullen and Robinson (4) have objected that both Warburg's formula and our empirical correction chart are ultimately dependent on the validity of Bohr's values for the solubility of  $\text{CO}_2$  in cells and plasma. This is a proper and serious criticism, especially when it is added that these solubility coefficients were determined on ox blood and not human blood and that Bohr assumed a mean value for cell volume in calculating the solubility coefficient in the blood cells.

It is quite possible to obviate Cullen and Robinson's criticism by developing an equation that will not involve  $\text{CO}_2$  solubility coefficients.

If we represent the concentration of total  $\text{CO}_2$  in blood, plasma, and cells by the symbols  $[\text{CO}_2]_b$ ,  $[\text{CO}_2]_p$ , and  $[\text{CO}_2]_c$ , respectively, and the cell volume by  $C$ , total blood volume being 1, then

$$[\text{CO}_2]_b = (1.00 - C) [\text{CO}_2]_p + C [\text{CO}_2]_c \quad (1)$$

In order to get an expression for  $[\text{CO}_2]_c$  in terms of  $[\text{CO}_2]_p$ , and for  $C$  in terms of hemoglobin, we may write

$$\frac{[\text{CO}_2]_c}{[\text{CO}_2]} = d \text{ or } [\text{CO}_2]_c = d [\text{CO}_2]_p \quad (2)$$

where  $d$  is the coefficient of distribution of  $[\text{CO}_2]$  between cells and plasma, and

$$\frac{h}{C} = q \text{ or } C = \frac{h}{q} \quad (3)$$

where  $h$  = the oxygen capacity of the whole blood and  $q$  is a factor indicating the relation of oxygen capacity to cell volume. Substituting for  $C$  and  $[\text{CO}_2]_c$  in equation (1), their equivalents from equations (2) and (3) we get the general equation

$$[\text{CO}_2]_b = \left(1.00 - \frac{h}{q}\right) [\text{CO}_2]_p + \frac{dh}{q} [\text{CO}_2]_p \text{ or inverting}$$

$$[\text{CO}_2]_p = \frac{[\text{CO}_2]_b}{1.00 - \frac{(1-d)h}{q}} \quad (4)$$



If the determinants of  $d$  and  $q$  could be established in terms of  $[\text{CO}_2]_b$  or a function of  $[\text{CO}_2]_b$ , this equation could be employed for the calculation of the carbon dioxide content of the plasma from the carbon dioxide content and oxygen capacity of the whole blood. Hamburger (5) long since showed that the cell volume varied with the hydrogen ion concentration of the blood. The observation has been repeatedly confirmed. It is evident that in any sample of blood a change in pH is associated with an alteration of the cell volume. Warburg, on the basis of this well recognized relation, decided that pH was the chief determinant of  $q$  and constructed a table of values of  $q$  at various levels of pH. The data which he used were derived from a limited number of experiments on horse blood in which variations of pH were produced by changing CO<sub>2</sub> tension. Now it is quite possible that a close relation between  $q$  and pH found under these conditions might be useless for the prediction of the value of  $q$  from pH in a series of samples of blood. The value of the differential,  $\frac{\Delta q}{\Delta \text{pH}}$  does not permit the prediction of the absolute value of  $q$  from pH.

In our own experiments, if  $q$  is compared with either pH or  $[\text{CO}_2]_p$  (see Columns 3, 5, and 6, Table III), as has been done in Chart 1, there is no evidence of any relation between them. However, in all our experiments in which determinations of cell volume and carbon dioxide content were made on samples of the same blood at different CO<sub>2</sub> tensions, there was a distinct tendency for the cell volume to increase with the CO<sub>2</sub> tension or hydrogen ion concentration. This is also true in certain experiments in which the change in hydrogen ion concentration was effected by adding lactic acid to the blood. Our work, therefore, is not at variance with that of previous observers. Evidently in a heterogeneous series of bloods collected from individuals under varying conditions of health and disease the relation of  $q$  to pH or CO<sub>2</sub> content, which is so definite in an individual specimen of blood, is hopelessly obscured by the introduction of other variable factors.

If one considers the blood from a morphological rather than from a chemical standpoint, it is hardly surprising that this should be so. The relation of the hemoglobin content and the number of red blood cells per unit volume of blood is fairly constant in normal individuals under standard conditions; it is highly variable in

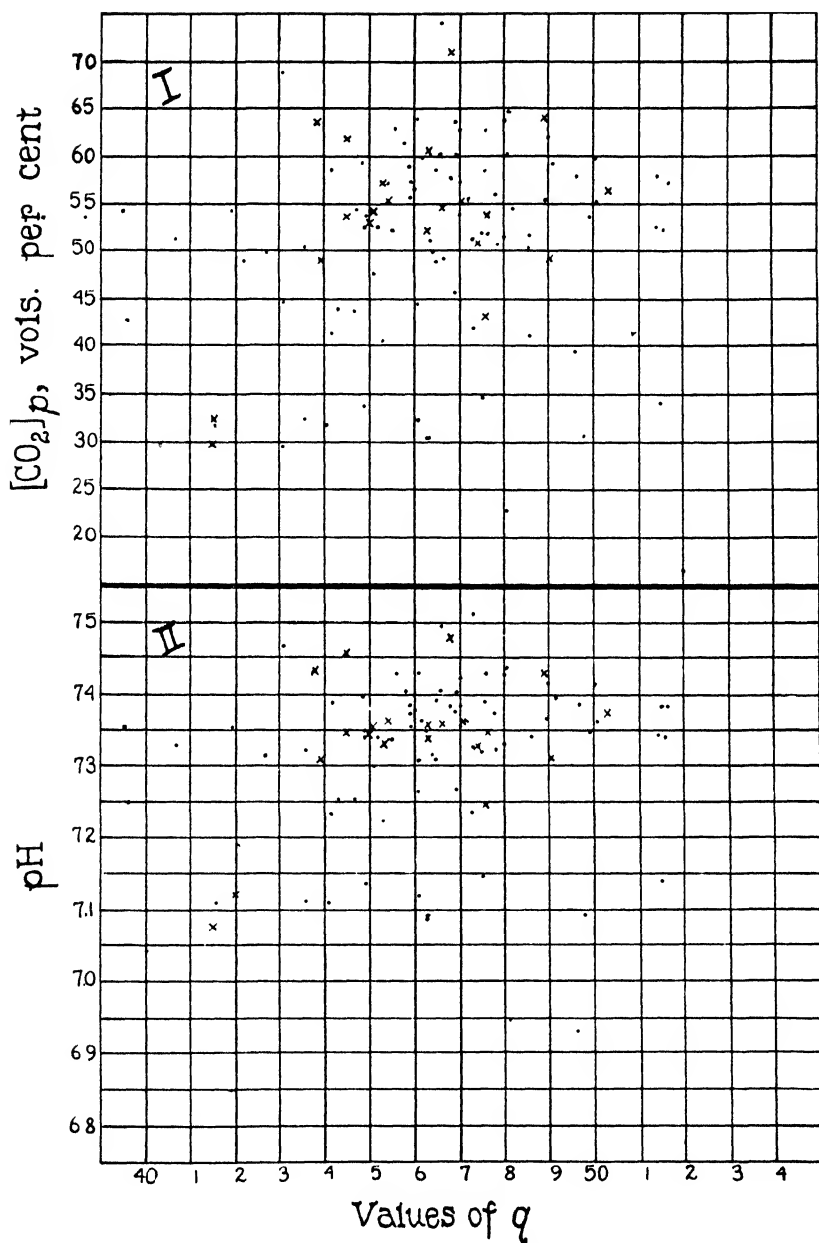


CHART 1. Abscissa = values of  $q$ ; ordinate for I =  $[\text{CO}_2]_p$ , vols. per cent, and for II = pH.

pathological states, and especially in anemias. It is inconceivable that these variations could be so exactly compensated by alterations in the size of the individual cells that they would not be reflected in the value of  $q$ . Unfortunately our data do not include red blood cell counts and therefore do not permit an evaluation of the effect on  $q$  of changes in the hemoglobin color index. Haden (9) has, however, published a series of figures for cell volume, red blood cell count, and hemoglobin values on normal and pathological persons that may be used for a comparison of color index and  $q$ .

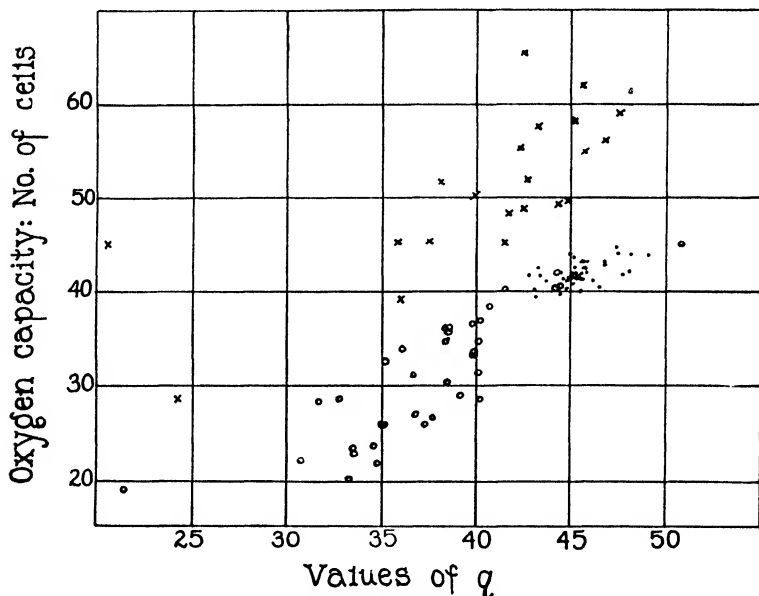


CHART 2 Abscissa = values of  $q$ , ordinate = ratio (oxygen capacity): (number of cells).

Such a comparison has been made from his data in Chart 2. In this chart the ordinate represents the color index in terms of the ratio (O<sub>2</sub> capacity in volumes per cent) : (the number of red cells, in 100,000); while the abscissa represents  $q$ , the ratio (O<sub>2</sub> capacity in volumes per cent) : (cell volume in volumes per cent). The dots represent observations on normal individuals, the crosses are from cases of pernicious anemia, and the circles from various types of secondary anemia. It is apparent first of all that there is a distinct tendency for the color index and  $q$  to change in the same

direction. This association holds for all types of pathological conditions investigated. At the same time, the pernicious anemia points show a systematic tendency to lie at a higher level than the others. This means that the individual red blood cells in pernicious anemia have a greater volume as well as a greater diameter than those found in normal persons or individuals suffering from secondary anemia.

In these studies Haden made no attempt to control the hydrogen ion concentration. Under these circumstances the fact that an undoubted relation between color index and  $q$  appears in Chart 2, while none can be found in Chart 1, proves that the actual value of  $q$  is not determined by the pH as much as it is by the condition of the individual. In this case  $q$  cannot be predicted, but must be determined in each blood specimen under standard conditions.

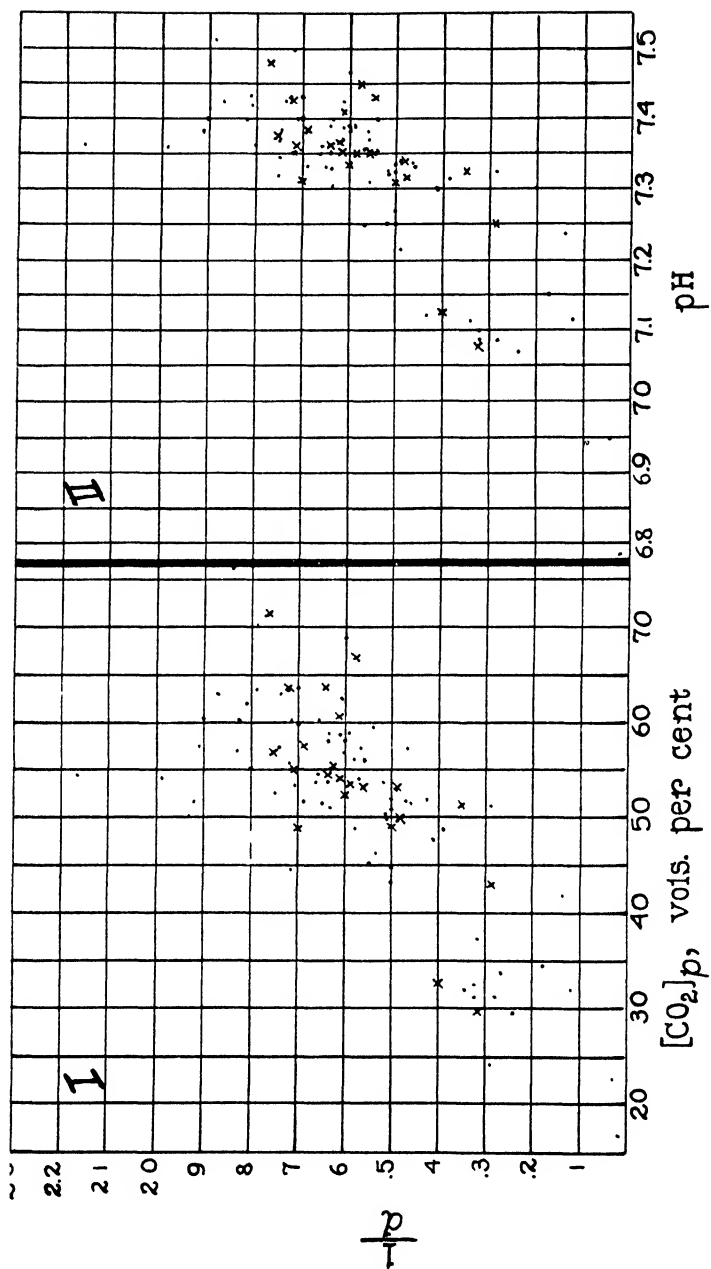
The distribution coefficient  $d$  of equations (2) and (4) can be determined easily from our data.

By combination of equations (1) and (2)

$$\frac{[\text{CO}_2]_b - [\text{CO}_2]_p (1.00 - C)}{C[\text{CO}_2]_p} = d \quad (5)$$

Column 7, Table III, gives values of  $\frac{1}{d}$ . In Chart 3 these have been plotted against both pH and  $[\text{CO}_2]_p$ .<sup>2</sup> The results support Warburg's statement that  $\frac{1}{d}$  increases with pH. The actual values obtained are not comparable with Warburg's because the latter are expressed in bicarbonate concentration instead of total  $\text{CO}_2$ . Although  $\frac{1}{d}$  increases with pH and with  $[\text{CO}_2]_p$ , the relationship is rough and the degree of scattering exhibited in the chart is so considerable as to render the chart more or less useless for the estimation of the differences between the concentrations of  $\text{CO}_2$  in plasma and in whole blood.

<sup>2</sup> The reciprocal of  $d$  has been used for two reasons. First, because it varies in the same direction as pH and  $[\text{CO}_2]_p$ , whereas  $d$  bears an inverse relation to the pH and the  $[\text{CO}_2]_p$ . In the second place values of  $\frac{1}{d}$  give more even distribution and, therefore, bring out the relation to pH and  $[\text{CO}_2]_p$  more clearly.



pH, ordinate =  $\frac{1}{d}$

vols. per cent and

C<sub>1</sub> RT Ab:

Although equation (5) which was used for the estimation of  $d$  is an accurate statement of the distribution of  $\text{CO}_2$  between the cells and the plasma, it implies nothing with regard to the mechanism that determines the inequality of this distribution. This inequality is, to a certain extent, only apparent.  $[\text{CO}_2]_p$  and  $[\text{CO}_2]_c$  indicate volumes per cent of  $\text{CO}_2$  in the total volumes of plasma and cells. The volume of water in the two phases of the system is, however, considerably diminished by the presence of other solutes and, especially, proteins. Ege (10), by studying the effect of glucose on freezing points, determined that the cells contained only about 65 per cent and the plasma about 93 per cent by volume of water. In other words about 35 per cent of the volume of the cells and about 7 per cent of the volume of the plasma were occupied by the proteins of the blood. The cells are known to contain about 35 per cent of hemoglobin and the plasma about 7 per cent of protein by weight. As the molecular weights and molecular volumes correspond so closely the former can probably be used as a measure of the latter without appreciable error for the calculation of the aqueous phases of the cells and the plasma.

$q$  is a measure of the concentration of hemoglobin in the cells when the former is expressed in volumes per cent of oxygen capacity. To convert this to percentage of hemoglobin by weight it is only necessary to divide by 22.4 and multiply by the molecular weight of hemoglobin or

$$\frac{q}{22.4} \times 1667 = [\text{Hb}] \quad (6)$$

where  $[\text{Hb}]$  is the concentration of hemoglobin by weight, or, if our assumption is correct, in volumes per cent, in the cells.

Then

$$100 - [\text{Hb}] = [\text{H}_2\text{O}]_c \quad (7)$$

In the same way

$$100 - [P] = [\text{H}_2\text{O}]_p \quad (8)$$

where  $[P]$  = the concentration of protein in the plasma.

Then

$$[\text{CO}_2]_{pw} = \frac{[\text{CO}_2]_p}{[\text{H}_2\text{O}]_p} \quad (9)$$

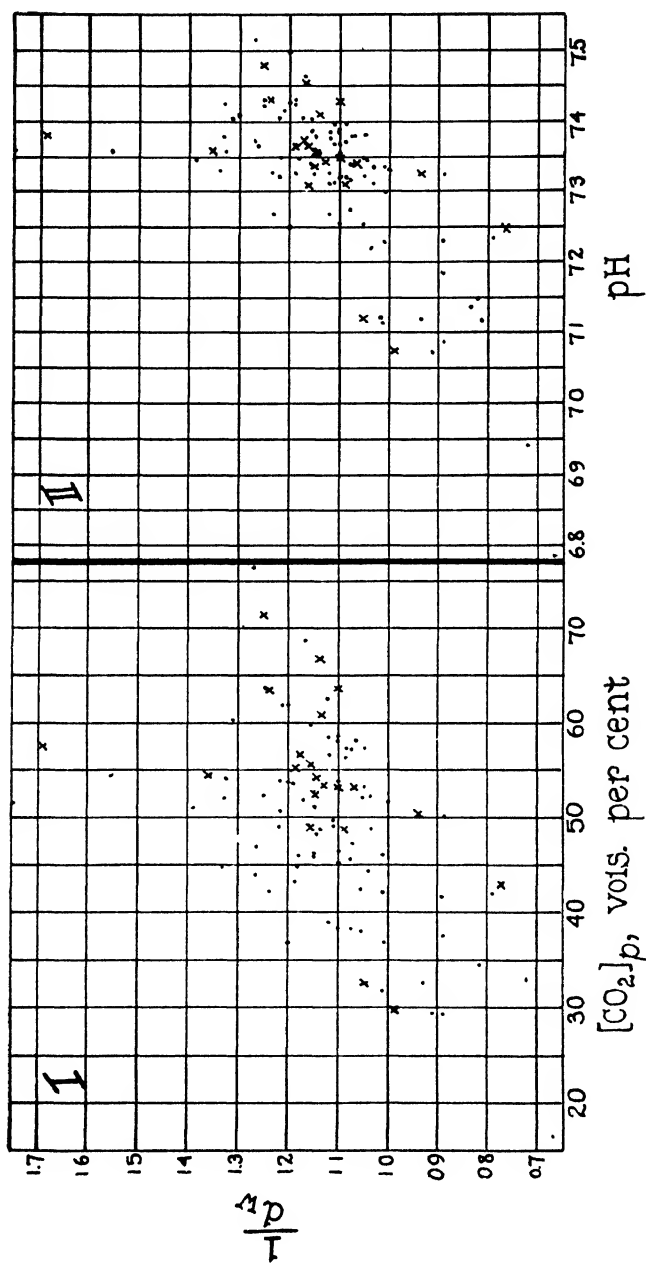


CHART 4 Abscissa for I =  $[CO_2]_p$ , vols per cent, and for II = pH; ordinate =  $\frac{1}{d_w}$ .

and

$$[\text{CO}_2]_{wv} = \frac{[\text{CO}_2]_c}{[\text{H}_2\text{O}]_c} \quad (10)$$

where the subscript *w* represents the aqueous phase.

By substitution

$$\frac{1}{d_w} = \frac{[\text{CO}_2]_p}{100 - [P]} \times \frac{100 - [\text{Hb}]}{[\text{CO}_2]_c} \quad (11)$$

$\frac{1}{d_w}$  has been computed for all the experiments and the results

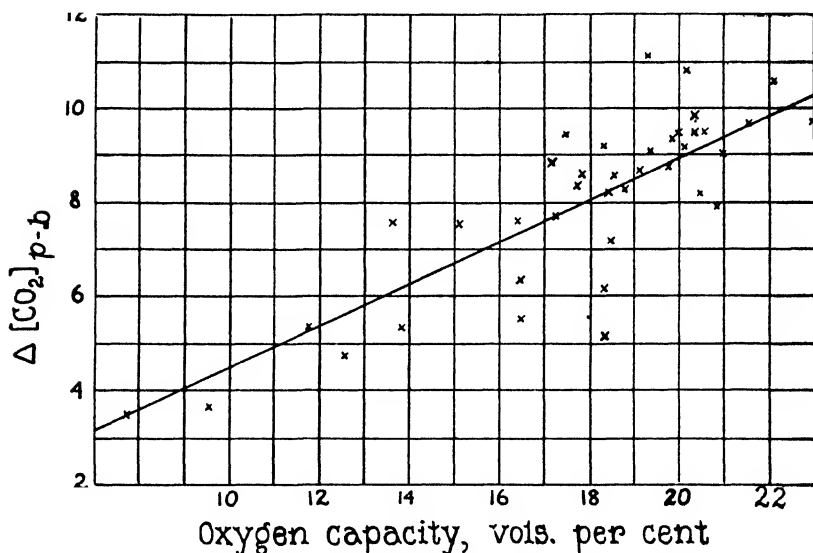


CHART 5. From experiments in which  $[\text{CO}_2]_p$  lay between 45 and 50 vols per cent. Abscissa = oxygen capacity, vols. per cent; ordinate =  $\Delta[\text{CO}_2]_{p-b}$ .

are shown in Column 8, Table III, and are plotted against pH and  $[\text{CO}_2]_p$  in Chart 4. There is no significant diminution of the scattering that was found in Chart 3. The causes of this scattering must therefore be sought in some other direction.

The impossibility of defining *q* and *d* in terms of carbon dioxide or hydrogen ion concentration offsets all the theoretical advantages of Warburg's method of correcting pH. From a practical point of view the method is quite complicated. If the aim is to predict



the CO<sub>2</sub> content of the plasma, and any method is bound to be empirical, the method which is most direct is to be preferred. Two of the most important factors that determine the difference in CO<sub>2</sub> content between blood and plasma are hemoglobin or cell content and the pH or bicarbonate content.

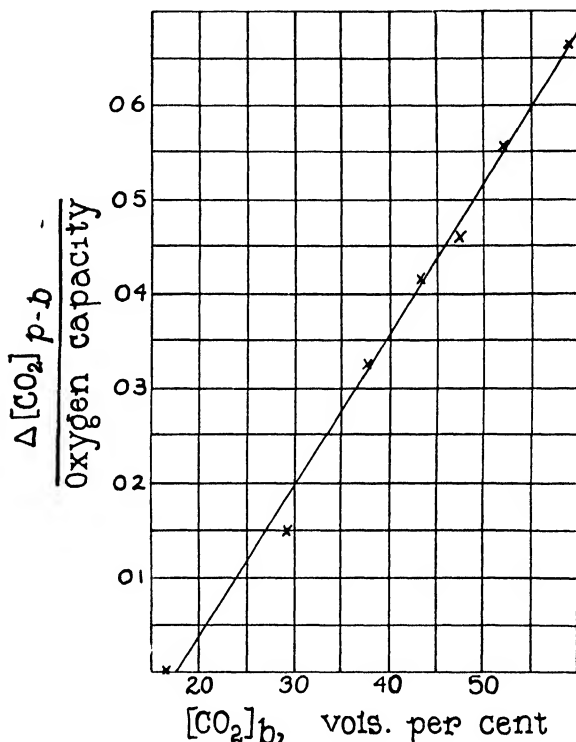


CHART 6. Abscissa =  $[\text{CO}_2]_b$ , vols. per cent, ordinate =  $\frac{\Delta[\text{CO}_2]_{p-b}}{\text{oxygen capacity}}$

In Column 10, Table III, may be found the simple observed difference between plasma and blood carbon dioxide, which has been called  $\Delta[\text{CO}_2]_{p-b}$ . In Chart 5 values of  $\Delta[\text{CO}_2]_{p-b}$  from blood samples in which  $[\text{CO}_2]_b$  lay between 45 and 50 volumes per cent have been plotted against corresponding oxygen capacities. There is evidently a relation between  $\Delta[\text{CO}_2]_{p-b}$  and oxygen capacity. As there is nothing in the arrangement of the observed points to

indicate that the relation is other than linear, the best straight line has been drawn through these points and the origin. The slope of this line is about 0.45.

Similar lines have been constructed for values of  $\Delta[\text{CO}_2]_{p-b}$  from specimens of blood with different values of  $[\text{CO}_2]_b$ . The results are given in Table I. As  $[\text{CO}_2]_b$  increases the ratio  $\Delta[\text{CO}_2]_{p-b} : h$ , the oxygen capacity, also increases. In Chart 6 the values of this ratio are plotted against  $[\text{CO}_2]_b$ . As  $[\text{CO}_2]_b$  increases the ratio

TABLE I

$[\text{CO}_2]_b$	No. of observations	Average $[\text{CO}_2]_b$	Average value of $\frac{\Delta[\text{CO}_2]_{p-b}}{h}$
<i>vols per cent</i>		<i>vols per cent</i>	
Below 20	1	16.3	0
20 to 30	6	27.6	0.153
30 " 35	6	31.6	0.147
35 " 40	11	37.7	0.324
40 " 45	22	43.0	0.421
45 " 50	41	47.3	0.459
50 " 55	19	52.2	0.554
Over 55	4	59.0	0.668

increases and the observed points fall close to a line defined by the equation

$$\frac{\Delta[\text{CO}_2]_{p-b}}{h} = 0.0159 [\text{CO}_2]_b - 0.281 \quad (12)$$

or

$$\Delta[\text{CO}_2]_{p-b} = (0.0159 [\text{CO}_2]_b - 0.281) h \quad (13)$$

But

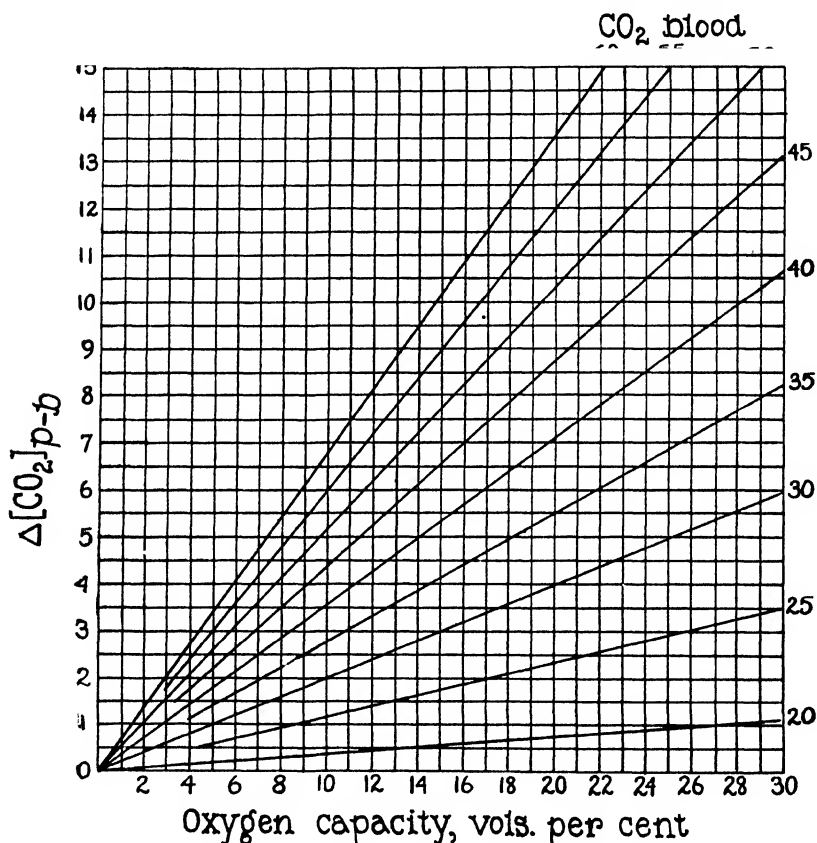
$$[\text{CO}_2]_p = [\text{CO}_2]_b + \Delta[\text{CO}_2]_{p-b}$$

Therefore

$$[\text{CO}_2]_p = [\text{CO}_2]_b + (0.0159 [\text{CO}_2]_b - 0.281) h \quad (14)$$

Equations (13) or (14) or Chart 7, which is a graphic representation of equation (13), can, therefore, be used to estimate the carbon dioxide content of plasma from that of whole blood at 40 mm. of  $\text{CO}_2$  tension.

The terminal qualifying phrase requires some explanation. It has been assumed by Warburg (6) that the effect of a change of pH on the distribution coefficient was the same whether that change was caused by altering CO<sub>2</sub> tension or altering fixed base of the blood. As we pointed out before (1) our data do not



CHAR 7.

entirely support this assumption. The matter is not an easy one to settle and attempts to measure the relative effects of the two factors have not proved entirely satisfactory. Nevertheless, they have convinced us that the point must be subjected to careful experimental analysis before a conclusion can be reached and studies along this line have already been instituted. Meanwhile,

TABLE II.

Experiment No	CO <sub>2</sub> tension.	O <sub>2</sub> capacity.	[CO <sub>2</sub> ] <sub>b</sub>	[CO <sub>2</sub> ] <sub>p</sub>	Δ[CO <sub>2</sub> ] <sub>p-b</sub> observed.	Δ[CO <sub>2</sub> ] <sub>p-b</sub> calculated.	Difference. Column 7 minus Column 6.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	<i>mm. Hg</i>	<i>vols per cent</i>	<i>vols per cent</i>	<i>vols per cent</i>	<i>vols per cent</i>	<i>vols. per cent</i>	<i>vols. per cent</i>
1	30	18 6	40 80	48 80	8 00	7 66	-0 34
	60	18 6	53 35	62 80	9 45	9 12	-0 33
2	30	12 4	23 75	25 70	1 95	2 01	+0 06
	60	12 4	33 90	36 50	2 60	1 78	-0 82
3	30	16 2	24 85	28 45	3 60	2 66	-0 94
	60	16 2	35 65	40 05	4 40	3 22	-1 18
4	30	22 4	39 23	47 30	8 07	8 50	+0 43
	60	22 4	52 80	63 60	10 80	11 09	+0 29
5	30	17 9	43 27	51 30	8 03	8 10	+0 07
	60	17 9	55 37	64 88	9 51	9 28	-0 23
7	30	16 1	35 68	38 38	2 70	5 43	+2 73
	60	16 1	46 15	50 50	4 35	5 88	+1 53
8	30	21 3	38 01	46 40	8 39	7 69	-0 70
	60	21 3	51 66	61 73	10 07	10 09	+0 02
9	30	16 5	42 38	47 80	5 42	7 30	+1 88
	60	16 5	53 62	61 46	7 84	8 02	+0 18
10	30	18 9	46 81	57 79	10 98	9 56	-1 42
	60	18 9	61 08	73 39	12 31	11 63	-0 68
11	30	21 3	39 69	48 66	8 97	8 27	-0 70
	60	21 3	53 36	63 98	10 62	10 65	+0 03
12a	30	17 8	42 40	50 20	7 80	7 80	0 00
	60	17 8	54 85	64 10	9 25	9 08	-0 17
b	30	17 1	43 47	51 85	8 38	7 83	-0 45
	60	17 1	56 61	66 20	9 59	9 17	-0 42
13a	30	23 6	48 44	60 43	11 99	12 34	+0 35
	60	23 6	62 82	77 69	14 87	15 51	+0 64
b	30	21 8	51 55	65 65	14 10	12 56	-1 54
	60	21 8	65 00	79 90	14 90	14 97	+0 07
14	30	19 2	44 79	52 77	7 98	9 09	+1 11
15	30	18 7	36 25	44 38	8 13	5 99	-2 14
	60	18 7	49 90	56 55	6 65	7 71	+1 06
16	30	17 5	47 25	55 60	8 35	8 63	+0 28
	60	17 5	60 05	69 15	9 10	9 84	+0 74
17	30	20 3	39 15	48 19	9 04	7 73	-1 31
	60	20 3	55 39	66 50	11 11	10 75	-0 36
18	30	18 3	41 40	46 70	5 30	7 71	+2 41
	60	18 3	53 15	57 85	4.70	8 89	+4 19
19	30	28 7	32 50	42 22	9 72	7 59	-2 13
	60	28 7	47 92	59 85	11 93	9 79	-2 14
20	30	20 0	39 70	47 85	8 15	7 81	-0 34
	60	20 0	52 10	62 20	10 10	9 52	-0 58
21	30	21 3	47 60	57 90	10.30	10 94	+0 64
	60	21.3	61 75	73 60	11 85	13 50	+1.65

TABLE III

O <sub>2</sub> capacity.	Cell volume	<i>q</i>	[CO <sub>2</sub> ] <sub>b</sub>	[CO <sub>2</sub> ] <sub>p</sub>	pH plasma (pK <sub>1</sub> = 6.1)	$\frac{1}{d}$	$\frac{1}{dw}$	Plasma proteins	Δ[CO <sub>2</sub> ] <sub>p-b</sub> observed. Column 5 - Column 4	Δ[CO <sub>2</sub> ] <sub>p-b</sub> calculated.	Difference Column 11 - Column 10.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
vols per cent	vols per cent		vols per cent	vols per cent				per cent	vols per cent	vols per cent	vols per cent
12 6	29 5	42 7	45 10	49 85	7 317	1 48	1 10	8 84	4 75	3 46	-1 29
9 9	21 4	46 3	29 30	30 75	7 091	1 28	0 89	5 76	1 45	1 87	+0 42
12 5	29 6	42 2	44 70	48 75	7 307	1 39	1 04	8 04	4 05	5 38	+1 33
14 3	31 9	44 8	41 70					5 54			
15 5	54 2	45 3	36 19	40 77	7 224	1 49	1 04	5 03	4 58	4 57	-0 01
18 7	41 5	45 1	42 06	47 78	7 298	1 41	1 01	7 69	5 72	7 26	+1 54
	39 2		39 90	46 12	7 281	1 53			6 22	6 42	+0 20
17 2	36 5	47 0	46 10	53 76	7 352	1 64	1 15		7 66	7 78	+0 12
17 5	36 6	47 8	47 00	56 40	7 374	1 83	1 27	6 73	9 40	8 17	-1 23
18 2	41 1	44 2	49 45	58 60	7 391	1 61	1 16	6 82	9 15	9 19	+0 04
18 7	43 0	45 8	49 07	60 18	7 404	1 82	1 30	7 21	11 11	9 33	-1 78
18 5	38 0	44 9	45 51	52 70	7 343	1 49	1 08	7 84	7 19	8 20	+1 01
20 0	44 3	46 5	41 23	48 91	7 309	1 58	1 12	7 62	7 68	7 50	-0 18
18 0	40 9	47 3	43 22	51 16	7 329	1 69	1 19	8 05	7 94	7 33	-0 61
18 0	36 2	40 7	42 23	51 13	7 329	1 65	1 24	7 20	8 90	7 04	-1 86
16 2	37 6	39 6	36 70	43 14	7 250	1 57	1 20	7 30	6 44	4 91	-1 53
17 2	39 6	47 6	53 60	63 30	7 427	1 73	1 19	5 95	9 70	9 82	+0 12
17 1	41 7	45 6	52 26	63 30	7 427	1 87	1 33		11 04	9 42	-1 62
19 1	39 9	48 2	45 90	54 56	7 359	1 66	1 15		8 66	8 58	-0 08
19 5	43 7	46 9	38 42	45 09	7 271	1 55	1 08	6 89	6 67	6 44	-0 23
18 2	38 3	45 5	45 99	52 13	7 338	1 42	1 01	7 38	6 14	8 21	+2 07
20 6	44 3	47 2	44 41	55 13	7 364	1 80	1 27	7 90	10 72	8 76	-1 96
17 8	35 9	46 6	62 27	71 15	7 498	1 72	1 20	6 68	11 88	12 63	+0 75
22 1	38 6	50 1	44 96	55 52	7 367	1 75	1 19	7 52	10 56	9 59	-0 97
16 1	42 9	44 7	38 18	43 40	7 253	1 50	1 06	5 01	5 22	5 25	+0 03
20 0	41 5	51 7	48 00	57 46	7 383	1 74	1 15	6 91	9 46	9 66	+0 20
21 0	36 7	49 0	46 40	55 40	7 366	1 61	1 10	6 93	9 00	9 60	+0 60
20 4	30 8	49 2	49 83	59 33	7 397	1 63	1 11	6 81	9 50	10 41	+0 91
17 6	43 4	48 0	53 70	63 50	7 428	1 72	1 20	7 30	9 80	10 08	+0 28
13 9	30 8	45 2	47 20	52 50	7 341	1 49	1 07	7 90	5 30	6 54	+1 24
20 2	43 4	46 6	49 30	60 10	7 403	1 71	1 22	8 15	10 80	10 15	-0 65
7 6	15 6	48 6	40 00	41 46	7 232	1 29	0 89	7 23	1 46	2 70	+1 24
7 2	15 7	46 1	31 47	32 70	7 102	1 32	0 93	7 28	1 23	1 58	+0 35
9 6	20 5	46 7	45 57	49 22	7 311	1 56	1 11	7 76	3 65	4 25	+0 60
7 8	16 4	47 5	46 91	50 37	7 322	1 75	1 22	7 98	3 46	3 63	+0 17
8 0	16 9	47 5	33 87	34 73	7 149	1 18	0 82	6 96	0 86	2 06	+1 20
19 7	38 3	51 5	31 47	34 22	7 142	1 27	0 84	7 56	2 75	4 31	+1 56
19 9	43 3	46 0	47 70	57 00	7 379	1 60	1 12	5 76	9 30	9 49	+0 29

TABLE III—Continued.

O <sub>2</sub> capacity.	Cell volume	<i>q</i>	[CO <sub>2</sub> ] <sub>b</sub>	[CO <sub>2</sub> ] <sub>p</sub>	pH plasma (pK <sub>1</sub> = 6.1)	$\frac{1}{\gamma}$	$\frac{1}{d\gamma}$	Plasma proteins	$\Delta[\text{CO}_2]_{p-b}$ observed Column 5 - Column 4	$\Delta[\text{CO}_2]_{p-b}$ calculated.	Difference Column 11 - Column 10.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
vols per cent	vols per cent		vols per cent	vols per cent				per cent	vols per cent	vols per cent	vols per cent
7 8	17 6	44 1	31 50	32 10	7 111	1 12	0 81	6 75	0 60	1 71	+1 11
6 4	14 7	43 6	30 95	32 40	7 116	1 43	1 02	5 47	1 45	1 36	-0 09
18 4	39 9	46 2	47 83	56 02	7 371	1 58	1 11	7 00	8 19	8 84	+0 65
14 9	28 9	51 6	47 40	52 25	7 339	1 47	1 04	5 13	4 85	7 05	+2 20
22 4	47 2	47 4	41 75	51 10	7 329	1 63	1 15	7 71	9 35	8 58	-0 77
21 3	45 1	47 3	60 80	76 50	7 512	1 84	1 27	6 01	15 70	14 60	-1 10
18 4	40 1	45 9	46 30	54 10	7 355	1 57	1 10	6 44	7 80	8 38	+0 58
20 1	46 2	43 6	42 85	50 62	7 324	1 50	1 09	7 38	7 77	8 06	+0 29
22 3	46 8	47 6	43 95	52 10	7 338	1 50	1 05	7 67	8 15	9 32	+1 17
11 0	25 4	43 1	28 14	29 59	7 072	1 24	0 91	7 88	1 45	1 84	+0 39
10 9	23 0	47 3	40 90	42 05	7 239	1 13	0 79	7 02	1 15	4 02	+2 87
9 4	22 5	41 6	30 17	32 01	7 110	1 34	1 01	7 64	1 84	1 87	+0 03
8 1	16 8	48 1	22 74	22 86	6 947	1 03	0 72	8 08	0 12	0 10	-0 02
18 8	40 0	47 0	49 10	57 40	7 382	1 57	1 09	6 96	8 30	9 40	+1 10
20 9	41 9	49 9	45 40	53 35	7 349	1 55	1 05	7 48	7 95	9 22	+1 27
19 2	37 4	51 4	44 25	52 75	7 343	1 75	1 16	6 49	8 50	8 12	-0 38
14 9	33 3	44 8	44 70	54 50	7 358	2 17	1 56	6 94	9 80	6 41	-3 39
13 7	28 4	48 1	52 12	60 20	7 404	1 90	1 31	6 90	8 08	7 49	-0 59
19 3	41 5	46 5	49 55	58 60	7 391	1 59	1 12	7 01	9 05	9 78	+0 73
22 6	48 0	47 0	51 38	62 81	7 423	1 61	1 12	6 77	11 43	12 10	-0 67
21 6	43 4	49 7	48 31	58 02	7 387	1 63	1 10	6 36	9 71	10 51	+0 80
20 3	43 2	46 9	54 45	63 55	7 428	1 79	1 25	7 01	12 10	10 82	-1 28
23 0	44 6	51 5	48 23	57 96	7 386	1 60	1 07	8 06	9 73	11 18	+1 45
19 8	43 2	45 9	47 40	56 15	7 372	1 56	1 09	5 96	8 75	9 37	+0 62
20 6	46 0	44 8	49 88	59 40	7 398	1 54	1 09	6 06	9 52	10 55	+1 03
21 3	49 6	43 1	56 00	68 81	7 465	1 60	1 17		12 81	12 97	+0 16
21 1	43 9	48 0	40 64	51 45	7 332	1 92	1 34	7 84	10 81	7 71	-3 10
13 7	28 0	48 7	46 58	54 17	7 356	1 99	1 33	4 47	7 59	6 29	-1 30
10 8	23 7	45 4	52 55	57 20	7 380	1 52	1 08	6 49	4 65	6 00	+1 35
19 7	39 4	50 1	50 22	59 90	7 402	1 70	1 16	8 13	9 68	10 18	+0 50
13 8	27 8	49 8	28 82	30 90	7 093	1 32	1 14	5 57	2 08	2 44	+0 36
15 2	30 9	49 0	53 50	62 13	7 418	1 81	1 21	5 24	8 63	8 67	+0 04
20 5	44 6	45 9	49 34	57 53	7 383	1 47	1 05	7 57	8 19	10 30	+2 11
20 1	42 1	47 6	49 35	58 53	7 391	1 59	1 10	6 99	9 18	10 15	+0 97
21 2	43 6	48 6	42 90	50 32	7 322	1 51	1 05	7 87	7 42	8 51	+1 09
17 8	38 5	46 1	53 70	63 80	7 430	1 70	1 19	6 17	10 10	10 20	+0 10
17 9	38 9	46 1	37 20	44 45	7 264	1 72	1 23	8 05	7 25	5 59	-1 66
15 1	33 7	44 9	45 95	53 51	7 350	1 72	1 22	6 03	7 56	6 80	-0 76

TABLE III—Concluded.

O <sub>2</sub> capacity.	Cell volume.	<i>q</i>	[CO <sub>2</sub> ] <sub>b</sub>	[CO <sub>2</sub> ] <sub>p</sub>	pH plasma (pK <sub>1</sub> = 6.1)	$\frac{1}{f}$	$\frac{1}{d\eta}$	Plasma proteins	$\Delta(\text{CO}_2)_{p-b}$ observed Column 5 - Column 4.	$\Delta(\text{CO}_2)_{p-b}$ calculated.	Difference, Column 11 - Column 10.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
vols per cent	vols per cent		vols per cent	vols per cent				per cent	vols per cent	vols per cent	vols per cent
23 3	44 7	52 0	16 28	16 46	6 779	1 02	0 67	5 69	0 18	-0 53	-0 71
20 3	42 1	48 1	52 50	64 70	7 437	1 81	1 25		12 20	11 25	-0 95
17 3	40 2	43 1	38 80	44 80	7 268	1 50	1 12	8 79	6 00	5 82	-0 18
17 9	40 5	44 2	36 80	41 50	7 232	1 39	1 01	7 92	4 70	5 44	+0 74
16 4	35 4	46 3	45 90	53 50	7 350	1 67	1 18		7 60	7 37	-0 23
14 3	32 3	44 3	38 62	43 45	7 254	1 52	1 10	6 66	4 83	4 77	-0 06
11 8	28 2	41 9	48 80	54 15	7 355	1 54	1 14		5 35	5 84	+0 49
20 1	43 3	46 4	42 60	49 85	7 317	1 51	1 08	8 50	7 25	7 99	+0 74
2 9	6 1	47 8	50 05	50 77	7 326	1 29	0 89	6 78	0 72	1 49	+0 77
19 5	42 0	46 3		30 90	7 093						
20 4	45 3	44 9		33 80	7 136						
3 6	11 0	32 4	50 86	53 30	7 348	1 71	1 39	7 18	2 44	1 90	-0 54
3 7	9 4	39 5	51 45	54 40	7 357	2 36	1 75	5 73	2 95	1 99	-0 96
22 5	48 0	46 8	44 55	57 75	7 385	1 91	1 33	6 61	13 20	8 62	-4 58
16 7	35 5	46 9	51 75	60 30	7 405	1 66	1 17	7 06	8 55	9 06	+0 51
16 5	34 1	48 6	46 15	51 65	7 334	1 46	1 00		5 50	7 48	+1 98
10 1	20 4	49 6	35 53	37 35	7 183	1 32	0 89	7 38	1 82	5 80	+3 98
			53 05	57 50	1 383				4 45		
18 6	41 2	45 1	45 59	54 16	7 355	1 62	1 15	7 15	8 57	8 27	-0 30
12 4	29 9	41 5	27 53	29 70	7 074	1 32	0 99	7 08	2 17	1 95	-0 22
16 2	38 6	42 0	29 15	32 79	7 122	1 40	1 05	5 30	3 64	2 97	-0 67
22 4	47 0	47 7	44 38	53 40	7 349	1 56	1 10	7 92	9 02	9 52	+0 50
17 9	35 6	50 3	47 92	56 53	7 375	1 75	1 17	6 91	8 61	8 61	0 00
16 1	33 8	47 6	39 70	42 98	7 249	1 29	0 77	6 19	3 28	5 64	+2 36
21 3	46 1	46 3	43 19	52 26	7 339	1 60	1 15	7 82	9 07	8 65	-0 42
16 5	36 7	45 0	46 71	53 04	7 346	1 48	1 07	7 76	6 33	7 62	+1 29
18 9	43 2	43 7	52 25	63 72	7 430	1 72	1 24	6 88	11 47	10 39	-1 08
21 3	45 7	46 7	44 84	54 48	7 358	1 63	1 36	7 12	9 64	9 07	-0 56
17 8	39 2	45 4	47 20	55 55	7 367	1 62	1 16	6 42	8 35	8 36	+0 01
17 1	37 7	45 3	48 50	57 36	7 382	1 69	1 69	7 21	8 86	8 38	-0 48
23 6	53 1	44 5	53 95	67 05	7 453	1 58	1 13	6 50	13 10	13 61	+0 50
21 8	46 5	46 8	56 74	71 20	7 480	1 77	1 25	7 29	14 46	13 53	-0 03
18 7	38 1	49 1	41 37	49 05	7 310	1 70	1 16		7 68	7 06	-0 62
17 5	37 7	46 3	52 16	60 86	7 409	1 61	1 13		8 70	9 59	+0 89
20 3	43 1	47 1	45 22	55 08	7 363	1 71	1 19		9 86	8 89	-0 97
18 3	38 7	47 4	45 95	51 04	7 328	1 35	0 94	6 44	5 09	8 24	+3 15
28 7	65 4	43 9	38 14	48 76	7 307	1 50	1 09	7 44	10 62	9 33	-1 29
20 0	45 0	44 5	44 42	53 33	7 348	1 59	1 13	6 17	8 91	8 50	-0 41
21 3	43 6	48 9	53 03	63 90	7 431	1 64	1 10	4 82	10 87	11 98	+1 11

if Warburg is right the present chart may be used for CO<sub>2</sub> tensions other than 40 mm. if a proper correction is made for the difference in dissolved CO<sub>2</sub>.

In Columns 10 and 11 of Table III, observed and calculated values of  $\Delta[\text{CO}_2]_{p-b}$  are compared. In Table II a similar comparison of values is given for specimens of blood at 30 and 60 mm. of CO<sub>2</sub> tension. There is a systematic difference in the error found at the two tensions. The error is not, however, significantly increased if the chart is used for any tension between 30 and 60, provided a proper correction is made for the difference in dissolved CO<sub>2</sub>. The CO<sub>2</sub> dissolved in plasma at 40 mm. = 2.85 volumes per cent. If then, equation (14) is to be used for tensions other than 40 mm. it must be altered to the form

$$[\text{CO}_2]_p = [\text{CO}_2]_b + \left\{ (0.0159 [\text{CO}_2]_b - 0.281) h + \left( \frac{P_{\text{CO}_2}}{40} \times 2.85 \right) - 2.85 \right\} \quad (15)$$

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## THE SPECIFICITY OF THE INTRACELLULAR HYDROGENASES IN FROG'S MUSCLE.

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Although we have evidence as to many of the intermediate products of metabolism, the details of the chemical changes and the nature of the enzymes bringing them about remain unsettled. A step forward was made in 1917 when Thunberg (1) suggested that the oxygen used by the tissues united with the hydrogen and not with the carbon of the food molecule, by aid of certain enzymes which he named dehydrogenases. This theory was based upon Wieland's observation that glucose could be oxidized by palladium in the absence of oxygen, the palladium acting both as catalyzer and hydrogen acceptor. When freshly ground tissue is allowed to act upon methylene blue in a vacuum, it behaves in similar fashion, giving up its hydrogen to the dye and so reducing it to the colorless leuco base. After repeated washing the tissue is no longer able to decolorize methylene blue without the addition of succinic acid or similar substances which alone cannot decolorize the dye. These substances ("donators") are also known to increase tissue oxidation, so it seems reasonable to regard them as intermediate products in tissue metabolism. Thus the evidence points toward a process of dehydrogenation (together with the addition and subtraction of water) as essential to tissue oxidations.

A single enzyme might conceivably catalyze the dehydrogenation process at every stage from glucose to  $\text{CO}_2$ ; or there might be a specific enzyme for every stage (2). The evidence on this question is not yet conclusive. The rate at which unwashed

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tissue can reduce methylene blue is very unequal in different animals and even in different tissues of the same animal. This inequality may be due to differences in kind and amount of food materials present in the tissues, to different enzymes, or to both. The washed muscle of frog or of goldfish can be activated by oxybutyric, succinic, or glutaminic acid as donator, so part of the oxidation process must be the same in both animals; though not all, for fumaric and maleic acids activate only the frog muscle (2, 3). This, of course, suggests the presence of specific enzymes which are similar in both animals for the early stages of oxidation but different for the later stages.

We know also that the treatment which a tissue undergoes alters its activity (2). Washing destroys first the power to oxidize formic and butyric acids; then citric, tartaric, and pyroracemic acids; then lactic and oxyglutaric acids; and finally succinic, glycerophosphoric, and ketoglutaric acids. Exposure to extreme cold ( $-80^{\circ}\text{C}.$ ) destroys spontaneous oxidation and activation by all donators except succinic and oxyglutaric acids. Between  $45$  and  $55^{\circ}$  the tissue progressively loses its power to oxidize glutaminic, then succinic, and finally glycerophosphoric acids. Is this because there are specific enzymes of unequal resistance, or because injury to one general enzyme hampers some processes more than others? We cannot be sure since the process which is least affected in every case is the oxidation of succinic or glycerophosphoric acid.

The only remaining bit of evidence is equally inconclusive. If an optimal amount of one donator is present in one tube and optimal amounts of two in another, the rate of oxidation should be the same or even a trifle slower if there is only one enzyme, but if there are two enzymes it should be more rapid in the second tube. Rosling's experiments (4) favored the two enzyme hypothesis, but Wishart (5) was unable to confirm his results. Wishart's experiments undoubtedly show that, if the donators are combined in pairs at concentrations which are optimal for each when acting alone, the rate of decolorization is equal to or slightly less than the rate for the faster donator acting alone; and this does not argue for the presence of more than one enzyme. In several cases at suboptimal concentrations, the addition of a second donator speeds up the reaction more than the addition of an

equivalent amount of the original donator; which certainly suggests the presence of more than one enzyme.

In the following experiments the attempt is made to distinguish the different enzymes, if they exist, through their resistance to various protoplasmic poisons.

### *Method.*

The skeletal muscle of a frog was quickly reduced to a pulp by snipping with curved scissors. The pulp was shaken for 15 minutes at room temperature with twenty times its weight of distilled water, filtered, and the process repeated once more. It was then weighed out in 0.2 gm. lots ready for use. Thunberg's vacuum tubes were used, having a capacity of 15 cc. with side arm and hollow stop-cock like that of a  $\text{CaCl}_2$  tube. The material in each tube consisted of:

Amount	
cc	
0 1	Methylene blue 1:5,000.
0 2	Buffer (6 6 per cent $\text{K}_2\text{HPO}_4$ plus 1 6 per cent $\text{KH}_2\text{PO}_4$ ).
0 1	Donator (citric acid, 0 2 M, or succinic acid, 0.05 M, neutralized with KOH).
0 0-0 6	Poison.
0 6-0 0	Water.
0 2 gm.	Washed muscle pulp.

making a total of 1 cc. plus the muscle. As soon as the muscle had been added the tube was evacuated with an ordinary water pump and placed in the water bath at  $35^\circ$ . The time was noted at which each solution was completely decolorized. Only winter frogs were used, but, of course, not all were in equally good condition, and small accidental differences in preparation also led to some day-to-day variation in the activity of the tissue. This, however, is not a serious difficulty, as both donators were tested on the same tissue with a given poison and the poisons were repeatedly tested on different days. Typical experiments are cited in Tables I and II.

## RESULTS AND DISCUSSION.

As Table I shows, the oxidation of citric acid in the presence of the washed tissue is more readily inhibited by chloral hydrate and by phenol than is the oxidation of succinic acid. But with other poisons (Table II) such as telurite, selenite, selenate, and arsenate, the oxidation of succinic acid is more readily inhibited than that of

TABLE I

*Effect of Poisons upon Washed Frog's Muscle as Evidenced by the Time Required to Decolorize Methylene Blue*

Poison	4 per cent solution of chloral hydrate		1 per cent solution of phenol		
	Citric	Succinic	Citric	Succinic	Glutaminic
cc					
0 0	35	40	63	60	75
0 1	38	50	150		
0 2	45	52	173	72	90
0 3	50	55	∞		120
0 4	∞	70	∞	95	∞
0 5	∞	100	∞		
0 6	∞			120	

TABLE II

Poison	<0.5 per cent telurite		0.2 per cent selenite		20 per cent selenate		10 per cent arsenate	
	Citric	Succinic	Citric	Succinic	Citric	Succinic	Citric	Succinic
cc								
0 0	40	90	20	23	80	90	40	60
0 1	50	∞	24	70	80	140	39	65
0 2	50	∞	40	140+			37	65
0 3		∞	44			200+	35	75
0 4	60		70+		210	∞	42	75
0 5							45	105
0 6							45	140

citric acid. If the succinic acid reaction were always more resistant than the other (as in the experiments on washing) we could not be sure whether two enzymes of unequal susceptibility were involved, or whether there was a single enzyme catalyzing both reactions but more efficient in one reaction than in the other.

Here, however, the case is much clearer, for the reaction which is more resistant to one set of poisons is more susceptible to the other set.

We might perhaps think of a single enzyme with a special "receptor" for each reaction, and conceivably these "receptors" might be unequally affected by the different poisons. But a simpler hypothesis, and one equally justified by the evidence, is that we have here two specific enzymes, one for the oxidation of citric acid and one for the oxidation of succinic acid. What more natural than that two specific enzymes should behave differently in the presence of poisons? This hypothesis of specific enzymes also fits in rather better with the observation that the earlier oxidation stages are similar in frog and fish muscle, while the later stages are unlike. Succinic acid is indeed oxidized by practically all tissues, so it seems reasonable to consider that an enzyme catalyzing this reaction is rather widely distributed. Wishart's observation that the donators in suboptimal concentrations work faster in pairs than alone also points to the existence of specific enzymes. We are therefore obliged to conclude that more than one enzyme is concerned in the long process of oxidation, and it is only the precise degree of specificity that is open to question.

#### SUMMARY.

Working with the methylene blue method, it is found that treatment with phenol or chloral hydrate destroys the power of washed frog muscle to oxidize succinic acid. The reverse is the case with certain other poisons such as telurite, selenite, selenate, and arsenate.

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# THE ACTION OF HYDRAZINE AND SOME OF ITS DERIVATIVES IN PRODUCING LIVER INJURY AS MEASURED BY THE EFFECT ON LEVULOSE TOLERANCE.

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Certain of the physiological properties of hydrazine have been studied by Underhill and his collaborators. The previous experiments of Borissow (1), Pohl (2), and Poduschka (3) had demonstrated the relatively great toxicity of this compound. Underhill and Kleiner (4) investigated the influence of hydrazine upon intermediary metabolism in the dog and found that hydrazine poisoning produces, among other symptoms, a condition of creatinuria with but slight alterations in the partition of other urinary constituents. Somewhat later, Underhill (5) presented data to show that doses of 50 mg. of hydrazine sulfate per kilo of body weight, when injected into dogs, lead to a distinct lowering of the sugar concentration in the blood. It is stated that subcutaneous injection of dextrose into dogs previously treated with non-fatal doses of hydrazine may cause the death of these animals. Underhill and Fine (6) have reported that glycosuria failed to manifest itself after pancreas extirpation in dogs which had received previous injections of hydrazine. According to these authors, animals treated with hydrazine and then depancreatized failed to develop hyperglycemia.

Later, Underhill and Murlin (7) showed that subcutaneous injection of hydrazine in fasting dogs induced an increased rate of carbohydrate combustion, this probably explaining the diminished blood sugar content as well as the disappearance of glycogen from the liver and muscles. More recently, Underhill and Bau-



mann (8) pointed out that in hydrazine poisoning the blood fat is markedly increased, the maximum being coincident with the hypoglycemia characteristic of the intoxication.

As in the case of phosphorus, hydrazine produces "fatty degeneration" of the liver. However, the effect of hydrazine differs from that of phosphorus in two particulars, according to Wells (9). Hydrazine attacks first the cells in the center of the lobule; phosphorus shows its first effects upon the peripheral cells. Phosphorus also causes fatty changes in the myocardium, kidneys, and other tissues; the effect of hydrazine is practically limited to the liver.

In a recent communication the writer (10) presented data, obtained in the case of a dog dying of hydrazine sulfate intoxication, in which the blood sugar gradually fell from 0.108 to 0.042 per cent during a period of 21 hours. The terminal hypoglycemia concomitant with death in this animal was similar in degree to that observed by the writer in severe phosphorus poisoning and by Mann and Magath (11) in dogs after liver extirpation. It was the writer's intention to employ hydrazine as a means of producing hepatic injury in connection with a study of the effect of experimental derangements of the liver on carbohydrate tolerance (12). Subsequently, the investigation was extended to certain of the derivatives of hydrazine. A number of these compounds proved to be relatively non-toxic, whereas other derivatives were found to be powerful protoplasmic poisons.

It is to be recalled that Underhill (13) made a brief study of the effect of certain hydrazine derivatives on the blood sugar concentration. Experiments were performed with methylhydrazine, phenylhydrazine, methylphenylhydrazine, diphenylhydrazine, and semicarbazide. In the case of methylhydrazine, Underhill states that the liver did not present the appearance characteristic of hydrazine poisoning. The injection of phenylhydrazine into a dog produced no unusual symptoms beyond the appearance of methemoglobin. Similarly, methylphenylhydrazine, diphenylhydrazine, and semicarbazide were found to be relatively non-toxic in their effect upon the liver. Underhill's experiments were of very short duration, usually not exceeding 2 days. No information is available, therefore, concerning the effect of prolonged or chronic poisoning with these compounds.

The writer believes that the derivatives of hydrazine may prove to be very useful for the experimental production of certain pathological conditions. In a number of preliminary experiments (unpublished), Hendrix and McAmis have observed a pronounced alkalosis in hydrazine poisoning. These workers are investigating the acid-base equilibrium of the blood in intoxications associated with liver tissue destruction due to hydrazine and other substances. The effect of certain hydrazine derivatives in producing anhydremia and experimental anemia will be reported in a forthcoming publication.

In the present investigation we have compared the effect of hydrazine and a number of its derivatives in producing liver injury. Among the compounds studied are the following: hydrazine (as the free base and as the sulfate), phenylhydrazine (as the free base and as the hydrochloride), acetylphenylhydrazine, methylphenylhydrazine, *p*-hydrazinobenzoic acid, diphenylhydrazine hydrochloride, symmetrical diisopropylhydrazine hydrochloride, and 2-2' azobispropane. Experimental evidence has been adduced to show that in conditions of liver involvement, as occur in chloroform and phosphorus poisoning, the tolerance for levulose may serve to indicate the degree of liver injury. In the following experiments, liver function was determined by this method, confirmation of liver damage being obtained by postmortem examination of the tissues. In the case of a number of animals, the utilization of glucose and galactose was also determined before and after poisoning. The procedure followed throughout was the same as that previously described (12, 14).

*Hydrazine  $H_2N-NH_2$ .*

In one experiment, a dog weighing 10.4 kilos, received 0.7 cc. of a hydrazine solution (hydrazine hydrate—Eastman Kodak Co.), containing 0.294 gm. of the free base. 2 days later there was definite indication of liver inefficiency, as shown by the data obtained in a levulose tolerance test (Table I). A second dose, similar in quantity, resulted fatally. Gross autopsy showed marked "fatty degeneration" of the liver. Microscopic examination of the liver showed extensive fatty degeneration with small areas of necrosis. Necrotic changes were likewise observed in the kidney cortex. The medullary portion appeared normal.

Very small doses of hydrazine sulfate may fail to cause serious impairment of the liver. This was shown in an experiment performed on Dog H-14 (Table II). The dog, weighing 8.8 kilos, received a subcutaneous injection containing 0.1 gm. of hydrazine sulfate. Similar doses were administered on the 6th and 9th days

TABLE I.  
*Effect of Hydrazine Poisoning on Levulose Tolerance.*

Dog H-29 Male

Day	Weight	Sugar administered	Blood sugar per 100 cc.				
			Initial	15 min	45 min	75 min	135 min.
	<i>kg</i>		<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
1	10 4	31 2 gm. levulose	87	90	96	108	94
2		Injected 0 294 gm hydrazine subcutaneously.					
4	10 0	30 0 gm levulose.	104	142	131	131	120

TABLE II  
*Carbohydrate Tolerance before and after Hydrazine Poisoning.*

Dog H-14. Male.

Day	Weight	Sugar administered	Blood sugar per 100 cc				
			Initial	15 min	45 min	75 min	135 min
	<i>kg.</i>		<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
1	8 8	26 4 gm levulose	98	115	121	112	103
2	8 8	26 4 " glucose.	98	141	126	107	107
3	8 8	26 4 " galactose	102	121	242	250	260
4		Injected 100 mg hydrazine sulfate subcutaneously.					
6	"	100 " "	"	"			
9	"	100 " "	"	"			
10	8 5	25 5 gm levulose	84	95	112	117	98
11		Injected 300 mg hydrazine sulfate subcutaneously					
12	"	300 " "	"	"			
13	8 0	24 0 gm. levulose	82	142	150	149	87
14	8 0	24 0 " glucose.	82	226	248	292	108
15	8 0	24 0 " galactose.	103	169	310	340	196
17	7 7	23 1 " levulose.	87	169	210	205	105

of the experiment. Normal liver function was observed at this time. Subsequently, the animal received two injections each containing 0.3 gm. of hydrazine sulfate. Whereas the smaller amounts of hydrazine produced no marked effect on liver function, the administration of the larger doses resulted in a lowered tolerance for

fructose as well as for glucose and galactose. The condition of the animal became progressively worse, as indicated by the test performed on the 17th day of the experiment. Autopsy, performed on the following day, showed fatty changes in the liver together with hemorrhagic areas. Microscopic examination of the liver tissue revealed extensive nuclear and protoplasmic degeneration.

TABLE III  
*Carbohydrate Tolerance before and after Hydrazine Poisoning.*  
Dog H-17 Female

Day	Weight	Sugar administered		Blood sugar per 100 cc					Remarks	
				Initial	15 min	45 min	75 min	135 min		
	kg			mg	mg	mg	mg	mg		
1	7.9	23.7 gm	levulose	80	102	100	98	95	0.395 gm hydrazine sulfate subcutaneously.	
2	7.9	23.7 "	glucose	100	141	214	214	115		
3	7.9	23.7 "	galactose	96	128	172	193	130		
4	7.6	22.8 "	levulose	111	122	159	189	180	0.370 gm hydrazine sulfate subcutaneously.	
5	7.5	22.5 "	glucose	98	103	200	161	107		
6	7.5	22.5 "	galactose	119	230	308	277	250		
8	7.7	23.1 "	levulose	113	165	137	114	92		
10	7.4	22.1 "	"	105	112	111	111	111		
11	7.0	21.0 "	"	98	125	137	127	120		
12	7.1	21.3 "	"	105	155	200	191	130		
15	Injected 700 mg hydrazine sulfate subcutaneously									
16	" 700 " " " "									
17				44 (Non-protein nitrogen 102)					Death occurred.	

Similar results were recorded in the case of Dog H-17 (Table III). A single dose of 0.395 gm. of hydrazine sulfate (50 mg. per kilo of body weight) caused a very pronounced decrease in liver function on the following day. Considerable improvement was noted several days later. At this time, a second injection of hydrazine sulfate was administered. As a result, the tolerance for levulose was again greatly diminished. For the purpose of producing extensive liver damage, two doses, each of 700 mg., were injected

on the 15th and 16th days. Death occurred on the 17th day of the experiment. Prior to death, the blood sugar had fallen to 44 mg. per 100 cc. The non-protein nitrogen had increased during the last day from the normal value of 40 to 102 mg. per 100 cc. of blood. This is believed to have been due, at least in part, to the excessive anhydremia which developed.

Microscopic examination showed diffuse fatty degeneration of the liver and almost complete disintegration of the hepatic cells. Despite the severity of the intoxication, the kidneys appeared normal. This accords well with the observations of Wells (9). It is to be noted, however, that hydrazine when administered as the free base appears to be less specific in its physiological action than when injected as the sulfate.

*Phenylhydrazine*  $C_6H_5 \cdot NH \cdot NH_2$ .

Hoppe-Seyler (15) showed that phenylhydrazine is very toxic, being especially destructive of the red corpuscles, due to its reducing effect on the hemoglobin. Phenylhydrazine reacts readily with aldehydes and ketones and is, therefore, a powerful protoplasmic poison. Only two experiments with this compound will be described in detail.

A dog weighing 5.9 kilos received four subcutaneous injections of an aqueous solution of phenylhydrazine hydrochloride, totaling 360 mg. of this compound. As a result, severe involvement of the liver was produced, as indicated by the data in Table IV. Very rapid destruction of the red corpuscles also occurred. The animal was killed on the 20th day of the experiment. On gross autopsy, the spleen was found to be greatly enlarged. Histological examination of the tissues showed marked hyperplasia of the spleen together with hematogenous pigmentation. Hematogenous pigmentation and extensive degenerative and necrotic changes were observed in the liver. Fatty changes were noted in the cortical portion of the kidney.

Corroborative data were obtained in a second experiment. Deficient liver function was manifest after the administration of a single dose of phenylhydrazine hydrochloride (150 mg.). Within 12 days, the red count had decreased from 5,640,000 per c. mm. to 910,000 (Table V). On autopsy, the spleen was found to be

about three times the normal size. Hyperplasia of the bone marrow was likewise noted. Slight fatty changes were observed in the liver. Microscopic examination revealed pigmentation, some

TABLE IV.

*Carbohydrate Tolerance before and after Phenylhydrazine Poisoning.*

Dog H-12. Female.

Day	Weight	Sugar administered	Blood sugar per 100 cc				
			Initial	15 min	45 min	75 min	135 min
	<i>kg</i>		<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg.</i>
1	5 9	17 7 gm levulose	106	134	126	116	97
2	5 9	17 7 " dextrose.	81	212	274	140	71
3	5 9	17 7 " galactose.	102	210	330	220	110
		Injected 80 mg phenylhydrazine hydrochloride subcutaneously.					
6		Injected 80 mg phenylhydrazine hydrochloride subcutaneously					
7		Injected 100 mg phenylhydrazine hydrochloride subcutaneously					
8		Injected 100 mg phenylhydrazine hydrochloride subcutaneously					
10	5 5	16 5 gm levulose	115	156	144	122	121
11	5 6	16 8 " dextrose	112	250	256	318	216
12	5 7	17 1 " galactose	95	190	320	291	160
		Injected 100 mg phenylhydrazine hydrochloride subcutaneously					
15	6 0	18 0 gm levulose	102	183	167	143	107
20	5 7	17 1 " "	90	160	190	174	100

TABLE V

*Levulose Tolerance before and after Phenylhydrazine Poisoning*

Dog H-26 Female

Day	Weight	Sugar administered	Blood sugar per 100 cc				
			Initial	15 min	45 min	75 min	135 min
	<i>kg</i>		<i>mg</i>	<i>mg.</i>	<i>mg.</i>	<i>mg</i>	<i>mg</i>
1	5 9	17 7 gm levulose	81	97	114	99	95
2		Injected 150 mg phenylhydrazine hydrochloride.					
5	5 9	17 7 gm levulose.	106	128	130	115	120
12	5 7	17 1 " "	97	141	152	197	121

fatty change and extensive regressive changes in the liver, hyperplasia of the spleen, and limited fatty degeneration in the kidneys.

Similar effects were produced on the administration of the free base. As in the preceding experiments, the onset of the anemia occurred rapidly. Hepatic inefficiency, due to destruction of liver tissue, was usually manifest.



Unsymmetrical methylphenylhydrazine appears to be less toxic than phenylhydrazine, both in its action on the liver and on the

TABLE VI  
*Levulose Tolerance before and after Methylphenylhydrazine Poisoning*  
Dog H-35. Female

Day	Weight	Sugar administered	Blood sugar per 100 cc				
			Initial	15 min	45 min	75 min	135 min
	kg		mg	mg	mg	mg	mg
1	19.1	57.3 gm levulose.	106	118	126	123	119
		Injected 0.2 gm methylphenylhydrazine					
5	19.0	57.0 gm. levulose	103	119	122	121	120
		Injected 0.5 gm methylphenylhydrazine					
7	"	1.0 "	"				
10	"	1.0 "	"				
11	19.0	57.0 gm levulose	110	113	116	122	117

blood. The administration of four doses of this compound, totaling 2.7 gm., failed to produce any noticeable effect on hepatic function in a dog, weighing 19.1 kilos (Table VI). On gross autopsy, the liver appeared normal, though somewhat enlarged. It appears from this and similar results that substitution of the unsymmetrical hydrogen atom in the phenylhydrazine molecule by an alkyl group, reduces the toxicity of the resulting compound.

*Diphenylhydrazine* ( $\text{C}_6\text{H}_5$ )<sub>2</sub> N · NH<sub>2</sub>.

A male puppy, weighing 4.2 kilos, received a single injection of an aqueous solution of diphenylhydrazine hydrochloride, containing 0.5 gm. of this compound. A similar or even much smaller dose of phenylhydrazine hydrochloride would have produced marked liver involvement. However, no such effect was ob-

served in this experiment (Table VII). On autopsy, the spleen appeared to be about normal in size. Congestion of the splenic capsule was noted. The liver seemed to be normal.

*Acetylphenylhydrazine*  $C_6H_5NH \cdot NHCOCH_3$ .

Acetylphenylhydrazine possesses marked reducing properties, though not to the same degree as phenylhydrazine, and resembles the latter compound in its destructive action on the blood (16).

TABLE VII

*Effect of Diphenylhydrazine Poisoning on Levulose Tolerance*  
Dog H-40. Male.

Day	Weight	Sugar administered	Blood sugar per 100 cc				
			Initial	15 min	45 min	75 min	135 min
	<i>kg</i>		<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg.</i>
1	4 2	12 6 gm. levulose	115	119	133	133	118
2		Injected 0 5 gm diphenylhydrazine hydrochloride					
4	3 9	11 7 gm levulose	107	125	119	116	102

TABLE VIII

*Effect of Acetylphenylhydrazine Poisoning on Liver Function.*  
Dog H-30 Male

Day	Weight	Sugar administered	Blood sugar per 100 cc				
			Initial	15 min	45 min	75 min	135 min
	<i>kg</i>		<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
1	10 5	31 5 gm levulose	84	104	106	105	104
		Injected 0 26 gm. acetylphenylhydrazine subcutaneously					
5	"	0 45 "	"		"		
9	9 9	29 7 gm levulose	80	106	108	111	108

In one experiment, a dog weighing 10 5 kilos, received two injections of acetylphenylhydrazine (0.71 gm). This produced no measurable effect on the liver, normal function being observed on the 9th day of the experiment. At this time, the dog was very anemic (Table VIII). The experimental results were confirmed on autopsy; very little destructive change of the hepatic cells being noted on microscopic examination of the liver tissue.



*p*-Hydrazinobenzoic Acid  $C_6H_4(COOH) \cdot NH \cdot NH_2$ .

*p*-Hydrazinobenzoic acid appears to be much less toxic than the hydrazine derivatives previously considered. Two injections of this compound (0.3 gm. each) had no effect on liver function, as indicated by the data recorded in Table IX. Subsequent administration of 2.2 gm. of this compound did not produce serious liver damage, as was shown at autopsy. It appears likely that *p*-hydrazinobenzoic acid is easily detoxified in the animal body.

TABLE IX

*Effect of p-Hydrazinobenzoic Acid on Liver Function*

Dog H-32 Female

Day	Weight	Sugar administered	Blood sugar per 100 cc				
			Initial	15 min	45 min	75 min	135 min
	<i>kg</i>		<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
1	9 4	28 2 gm levulose	105	113	118	128	97
2	Injected 0 3 gm <i>p</i> -hydrazinobenzoic acid *						
3	9 4	28 2 gm levulose	104	114	121	110	115
4	Injected 0 3 gm <i>p</i> -hydrazinobenzoic acid *						
5	9 4	28 2 gm levulose	109	130	133	133	105

\* Dissolved in sodium carbonate

*Symmetrical Diisopropylhydrazine*  $(CH_3)_2CH \cdot NH \cdot NH \cdot CH \cdot (CH_3)_2$ .

This compound, synthesized by Lochte, Bailey, and Noyes (17), was furnished by these authors for the present work. In a previous communication, Bodansky and Hartman (18) pointed out that this compound is destructive both of liver tissue and red corpuscles. Definite disturbance of liver function was observed following the administration of 0.258 gm. of this compound to a dog weighing 12.9 kilos. The condition became more aggravated after subsequent injections of the same substance, as indicated by the data presented in Table X.

Gross autopsy showed enlargement of the spleen, fatty change in the liver, and hyperplasia of the bone marrow. Microscopic examination revealed hematogenous pigmentation and fatty degeneration of the liver.

A rabbit, weighing 1.56 kilos, received 39 mg. of symmetrical diisopropylhydrazine hydrochloride. On the following day, a second dose of 70 mg. was injected. Death occurred 2 days later. Histological study of the tissues showed hyperemia of the spleen and destructive changes of the parenchyma in the kidney cortex. Extensive disintegrative changes of the hepatic cells were likewise noted.

*2-2'Azobispropane*  $(CH_3)_2CH \cdot N \cdot N \cdot CH(CH_3)_2$ .

2-2' Azobispropane was prepared by the oxidation of symmetrical diisopropylhydrazine hydrochloride according to the

TABLE X  
*Effect of Symmetrical Diisopropylhydrazine Hydrochloride Poisoning on Liver Function.*

Dog H-21 Male

Day	Weight	Sugar administered	Blood sugar per 100 cc				
			Initial	15 min	45 min	75 min	135 min
	<i>kg</i>		<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
1	12.9	38.7 gm levulose.	96	113	119	119	101
		Injected 0.258 gm symmetrical diisopropylhydrazine hydrochloride					
	12.9	38.7 gm levulose	108	145	133	125	108
	12.5	37.5 " "	111	170	136	127	120
	12.4	37.2 " "	105	121	159	153	116
	12.2	36.6 " "	103	141	143	141	116
		Injected 0.120 gm symmetrical diisopropylhydrazine hydrochloride					
		Injected 0.240 gm symmetrical diisopropylhydrazine hydrochloride					
11	11.5	34.5 gm levulose	102	143	157	170	127

method of Lochte, Noyes, and Bailey (19). A dog, weighing 6.7 kilos, received a single subcutaneous injection, containing 2 cc. of the azobispropane. Death occurred the same night. Microscopic examination of the tissues showed acute diffuse nephritis, almost hemorrhagic in nature. Hyperemia and parenchymatous degeneration of the hepatic cells were noted. The cell nuclei took the acid instead of the basic stain.

In a subsequent experiment, a smaller dose was administered (0.5 cc.). Liver inefficiency was observed 2 days later. At this

time, Cheyne-Stokes respiration was observed. There was also an increase in the non-protein nitrogenous constituents of the blood. The dog died the same day. Autopsy showed parenchymatous degeneration of the pancreas, fatty degeneration in the liver, and acute destructive changes in the cortical portion of the kidney.

The effect of even smaller doses of 2-2' azobispropane was determined in another experiment. Following the subcutaneous injection of 100 mg. of this compound, emulsified in olive oil, marked increase both of the hemoglobin concentration and of the red cell

TABLE XI  
*Effect of 2-2' Azobispropane on Liver Function*

Dog H-41. Female.

Day	Weight	Sugar administered	Blood sugar per 100 cc				
			Initial	15 min	45 min	75 min	135 min
	<i>kg</i>		<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
1	6 7	20 1 gm levulose.	102	105	107	105	100
		Injected 100 mg azobispropane in olive oil subcutaneously.					
3	6 6	19 8 gm levulose	102	127	137	143	127

count was noted. This was obviously due to anhydremia. 2 days after the injection, the function of the liver was deranged, as shown by the data in Table XI. The dog recovered within the next 2 weeks.

From the results of these and similar experiments, it is to be concluded that 2-2' azobispropane is a very toxic protoplasmic poison.

#### SUMMARY.

A study has been made of the action of hydrazine and certain of its derivatives in producing liver injury.

The levulose tolerance test, previously described, was employed in determining the degree of liver involvement. This method has been found to be very useful, as shown by the corroborative evidence adduced on gross and microscopic postmortem examination of the tissues of the experimental animals.

The results obtained with hydrazine are in close agreement with the observations of earlier investigators. In addition to its action

on the blood, phenylhydrazine is very destructive of liver tissue. Symmetrical diisopropylhydrazine (hydrazo-2-propane) and 2-2' azobispropane are powerful poisons and are especially injurious to the liver. Certain derivatives of hydrazine, such as methylphenylhydrazine, diphenylhydrazine, acetylphenylhydrazine, and *p*-hydrazinobenzoic acid, are much less effective in this regard.

In conclusion, the writer wishes to express his indebtedness to Professor J. B. Sumner in whose laboratory this work was completed and to Professor H. C. Hartman who made the microscopic diagnoses.

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## THE RATES OF REDUCTION AND OXIDATION OF BLOOD.

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The transportation of oxygen from the lungs to the tissues is one of the fundamental physiological processes. The factors involved in this problem are many; such as the efficiency of pulmonary ventilation, disturbances of the circulatory system or the phases governed by the demand and supply of the oxygen in the tissues and atmospheres, respectively. One aspect of this problem has been especially well worked out; namely, the carrying capacity of blood for oxygen at various partial pressures of oxygen and the effect of acids, bases, and salts thereon. For this knowledge we are indebted to the highly productive efforts of Barcroft, Bohr, Hasselbalch, and Krogh. This work, although of the greatest value, deals with the problem as a static one and the oxygen dissociation curve is one of final equilibrium. The dynamic aspect of this equilibrium, however, is probably of even greater importance and the following discussion and work have been undertaken from this standpoint.

At the alveolar oxygen tension of approximately 100 mm. of Hg the factors, such as hydrogen ion concentration and the variation of salt content, which deflect the body of the dissociation equilibrium curve, are not effective in appreciably altering the total oxygen capacity of the blood. It is apparent, however, that it is highly important to know whether or not the rate of the oxidation of the blood as it races through the pulmonary capillaries is sufficiently rapid under various conditions of the blood, to saturate it completely. The question of the rapidity of reduction in the tissue is equally important.

Previous studies upon the velocities of these reactions were made in Barcroft's laboratory during the years 1909 to 1912

before the method for determining the hydrogen ion concentration was developed by Hasselbalch for accurate use with whole blood. Barcroft and Hill (1) studied the rate of reduction of an oxyhemoglobin solution by bubbling a stream of nitrogen through it and found that the rate of deoxygenation obeyed the mass law. Mathison (2) encountered trouble when he attempted to apply the same method to whole blood because of the marked tendency to froth. He succeeded, however, in devising a method of breaking up the bubbles formed and obtained some interesting data by passing nitrogen bubbles through various bloods at a uniform rate. He showed that inorganic and organic acids increased the rate of dissociation proportional in the main to the estimated change in hydrogen ion concentration produced. Oinuma (3) used the apparatus of Mathison and showed that the respective rates of reduction and oxidation were approximately the same under physiological conditions—the presence of 40 mm. of  $\text{CO}_2$  pressure accelerated the rate of reduction and depressed the rate of oxygenation. It is remarkable that in spite of the lack of any great degree of physical constancy, consistent results were obtained from a method so empirical.

In an attempt to ascertain the effect of changes in hydrogen ion concentration and especially as to whether or not  $\text{CO}_2$  had a specific effect on the rates of oxidation and reduction, the method of bubbling gas mixtures through blood was tried. It was discarded, however, because of the lack of physical constancy and because the periods of reduction and oxidation extended over too long a period, an hour or so, instead of a few minutes. Hemoglobin, especially at the higher pH values, changes in respect to oxygenation and reduction at body temperature over extended periods of time. A new method to overcome these difficulties was therefore sought for.

### *Methods.*

An attempt was first made to measure directly the increase in volume of nitrogen-carbon dioxide mixtures when exposed to oxygenated blood. Conversely, it was expected to measure the decrease in volume of an oxygenated  $\text{CO}_2$  mixture when exposed to reduced blood. The apparatus used is shown in Fig. 1. 2 cc. of blood, which had first been equilibrated with 100 mm. of

Hg oxygen tension and a carbon dioxide tension equal to the nitrogen reducing mixture, were run from the side arm *A* into the flask *B* of 150 cc. capacity which had first been filled with the reducing gas mixture. The apparatus had previously been suspended in a constant temperature water bath regulated to  $\pm 0.01^\circ$  C. using points *C* and *D* at the extremities of the horizontal arm as bearing supports. A pitman connected to the top arm at point *E* permitted the flask *B* to be rapidly oscillated at a uniform rate

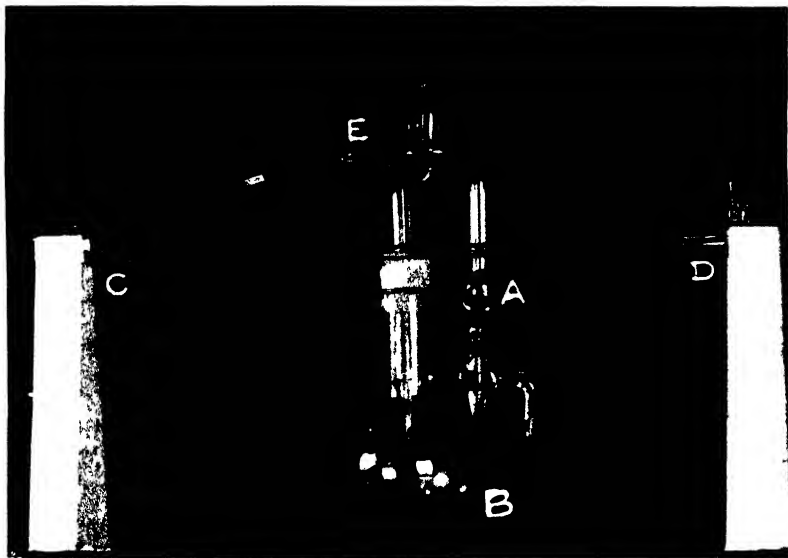


FIG. 1

and thus spread the blood in continually fresh forming surfaces over the inside of the flask. The change in position of a droplet of mercury in the horizontal tube *CD* which was graduated to 0.01 cc. gave the change in volume of the gas mixture due to the oxygen given off or taken on by the blood. By reading the mercury meniscus every half minute or by photographing it on a moving strip of sensitized paper, a curve was formed showing the velocity and extent of the oxidation or reduction reaction. By using perfectly clean mercury and a clean but moist tube together with the oscillating rotation of the tube, a very accurate record of volume changes could be obtained.



The method, however, was not applicable to the problem but the explanation was not apparent although very obvious, until many attempts had been made. The work of Christiansen, Douglas, and Haldane (4) in 1914 showed that oxygenated blood absorbs less  $\text{CO}_2$  at a given tension of that gas than does reduced blood at the same  $\text{CO}_2$  tension. The application of the Hasselbalch (5) formula to this observation

$$\text{pH} = \text{pK}_1 + \log \frac{\text{NaHCO}_3}{\text{H}_2\text{CO}_3}$$

would warrant the assumption that there is a pH change toward the acid side as reduced blood becomes oxygenated. That this is the case was shown to be true by Parsons (6) in 1917 by direct pH measurements on blood plasma. He found the difference between oxygenated and reduced blood to be about 0.038 of a pH unit which at a constant  $\text{CO}_2$  tension is equivalent to about 4 volumes per cent of combined  $\text{CO}_2$ . It was this magnitude of error which was apparent in our change of volume determinations for as the blood became deoxygenated it would not only give up 18 volumes per cent of oxygen but it would also take up approximately 4 volumes per cent of  $\text{CO}_2$  so the total volume change was only 14 volumes per cent. Parsons stated that the pH change upon reduction was a constant one regardless of the pH range as determined by various  $\text{CO}_2$  tensions. This apparently holds only when the pH is changed by  $\text{CO}_2$  tensions, but the pH change is of a varying magnitude when the range is changed by the addition of acids as lactic or by bases. A paper at a later date will present this point more fully. These discrepancies rendered the above discussed method impractical.

#### *Colorimetric Method.*

It seemed possible that the color change accompanying the reduction or oxidation of blood might be used as an index determining the oxygen content at any one moment. Haldane (7) had already determined the total oxygen capacity of various bloods by color comparison and found that it checked favorably with the ferricyanide method. This led us to compare partially and completely reduced blood with fully oxygenated blood in a Bausch and

Lomb colorimeter and it was found that the color quality by transmitted light is the same so that a perfect match can be obtained. Reduced blood was found to have about twice the color density of

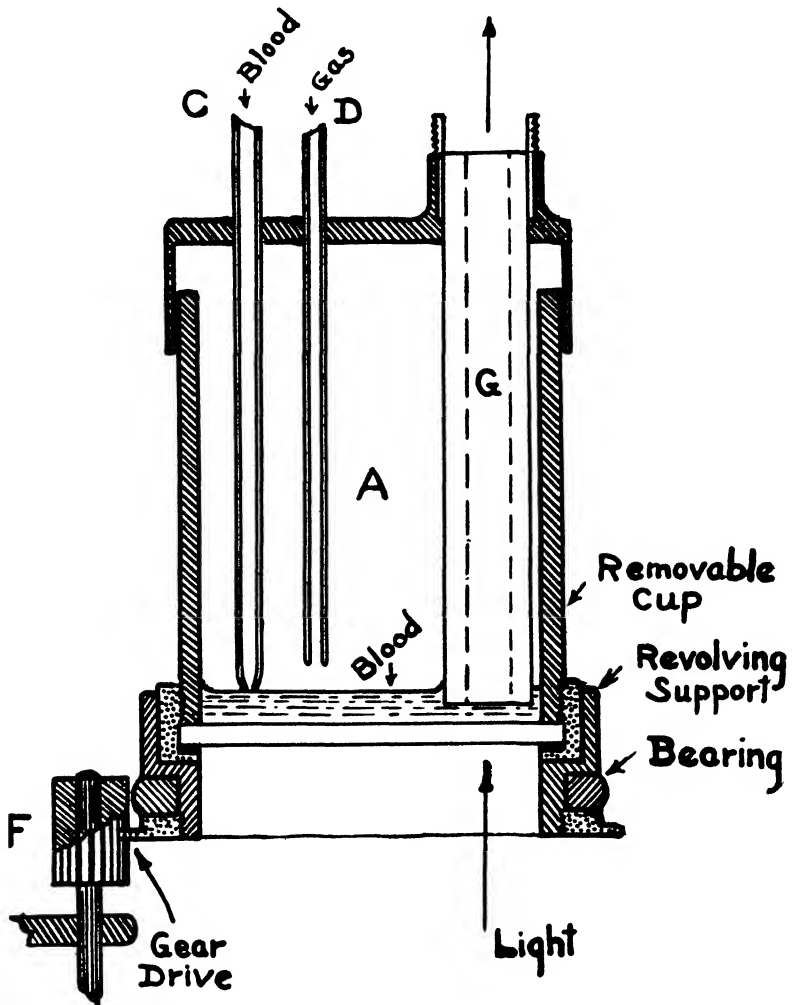


FIG. 2.

fully oxygenated blood. An apparatus was then devised, Figs. 2 and 3, using a Bausch and Lomb colorimeter wherein one of the cups (A) revolved eccentrically around the glass column

(G). In this way the blood was stirred and a continually changing amount of blood passed under the observing column. In the closed portion of the cups over the blood various gas mixtures could be passed through the tube (D) which extended down to within 3 mm. of the surface of the blood. The cup, the cross-section of which is shown in Fig. 2 was 45 mm. in diameter and was driven by means of a positive gear drive *F* (Figs. 2 and 3) at

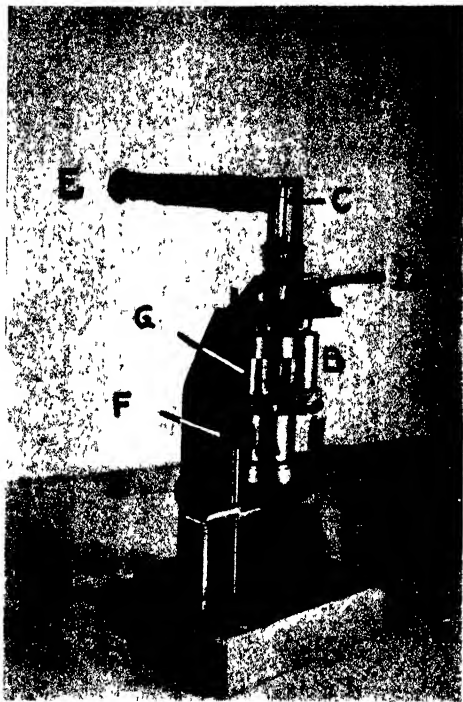


FIG 3

a constant speed of 165 to 170 revolutions a minute. The bearing upon which the cup revolves must be machined extremely accurately and it must run perfectly true as otherwise there will be a variation in the depth of the observed blood at each revolution of the cup. Although this variation may be extremely small, the resulting flickering of the color field makes accurate matching impossible. The vertical movement of the revolving cup (A) and hence the depth of the observed blood as regulated by the rack

and pinion screw (*R*) Fig. 4 was greatly magnified and recorded by means of a lever (*L*) on a smoked drum (*K*). This complete set-up is shown in Fig. 4. The extent of magnification and the method of recording can be seen by the vertical lines representing mm. of depth on the kymograph record, Fig. 5.

The complete apparatus was set up in a constant temperature air bath which was kept at  $37.5 \pm 0.1^\circ\text{C}$ . This arrangement is shown in Fig. 6. In determining the rate of reduction of fully

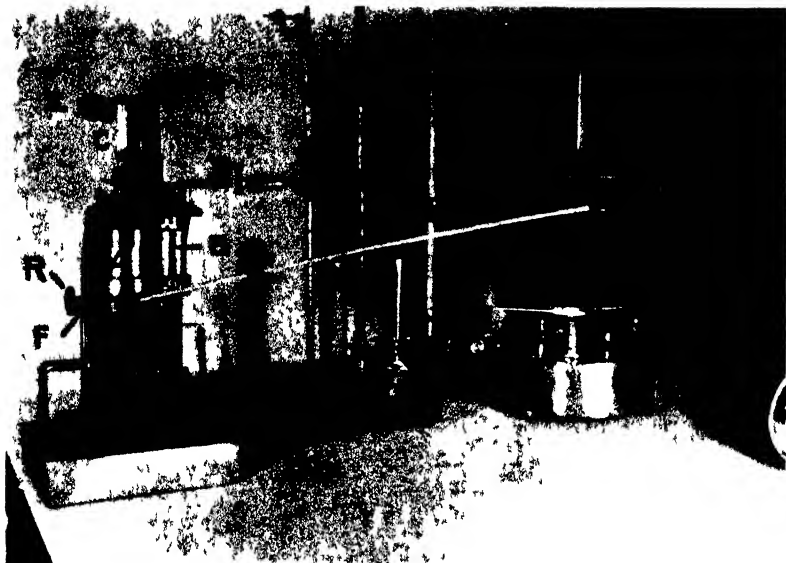


FIG. 4

oxygenated blood, two 2 cc samples of the same blood are placed, one in the stationary cup (*B*) set at a standard depth of 4 mm. and the other in the receptacle (*C*) closed by the stop-cork. Previously the revolving cup (*A*) which has been set at approximately 4 mm. has been filled with a nitrogen or nitrogen-carbon dioxide mixture saturated with water vapor at  $37.5^\circ\text{C}$ . through the tube (*D*). A small amount of the nitrogen mixture is kept flowing into the cup to prevent diffusion of atmospheric oxygen into the cup at its junction with the cap. The blood from the cup (*C*) is then run into the revolving cup and the color of the two fields of the colorimeter as observed at the eye-piece (*E*) is matched by

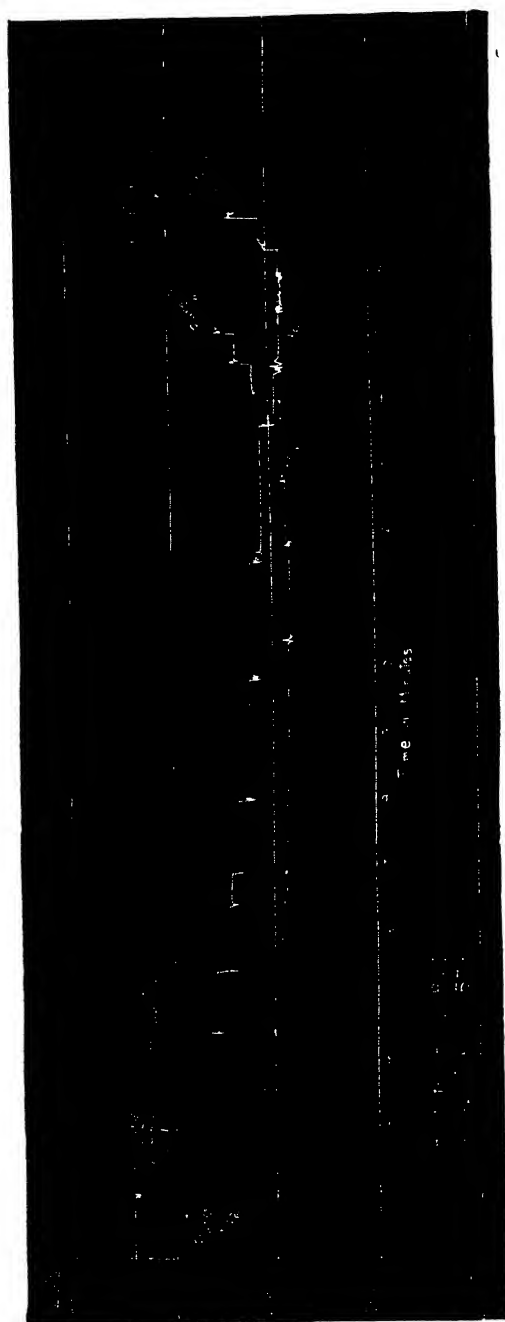


Fig. 5.

varying the height of the cup (*A*) by means of the rack and pinion screw (*R*), Fig. 4. This depth is recorded on the revolving smoked drum. The blood upon becoming deoxygenated becomes denser in color and the cup is moved up as the fields are again matched. This process is continued until there is no more color change and the curve of the rate of reduction is recorded upon the drum. Such a curve is shown in Fig. 5. Similarly, fully reduced blood in contact with oxygen mixtures can be compared with oxygenated blood and a reverse curve obtained.

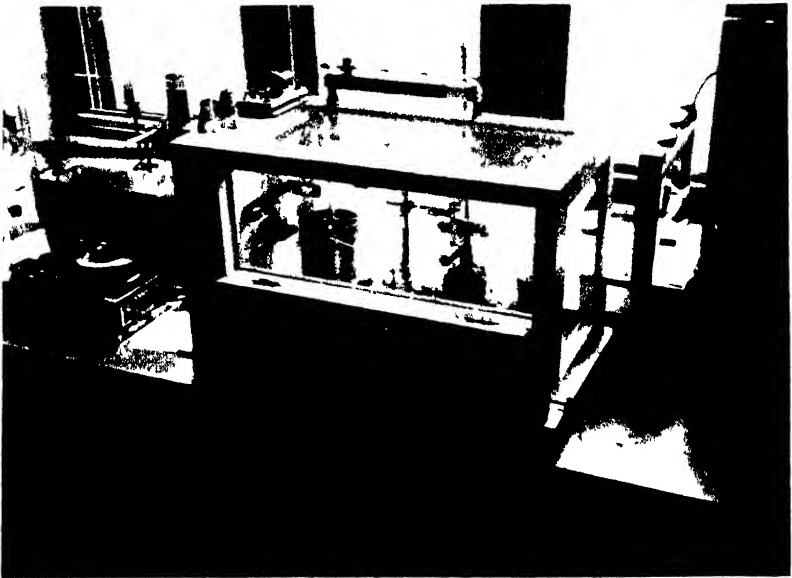


FIG. 6

## EXPERIMENTAL.

Fig. 5 shows a kymograph record of one of the runs. All the curves presented were transposed from such original records. The steps in the record show the levels after the two fields were matched. A comparison was made about each half minute. The oscillations before each step represent the attempts to match the fields. In transposing the curves, the points that were measured from the base line were taken right after the fields were matched. The depth of the blood in the standard cup was always

set at a 4 mm. depth. This depth with a 1,000 watt light and condenser gave a good illumination for comparison of the colors.

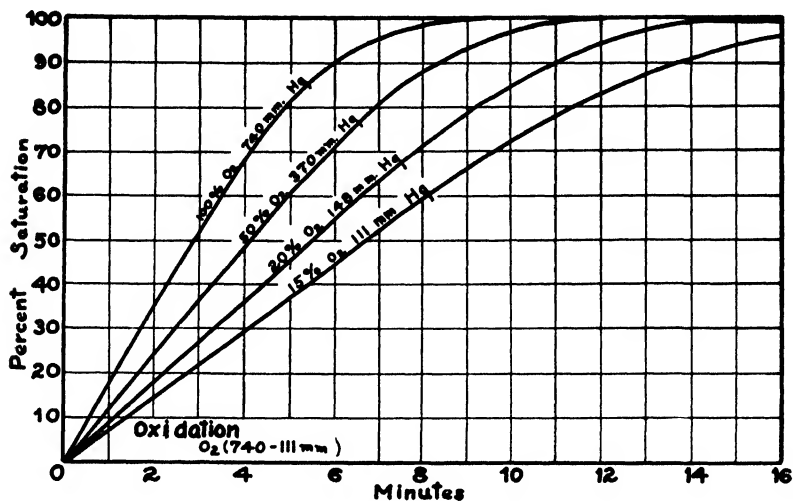


CHART 1.

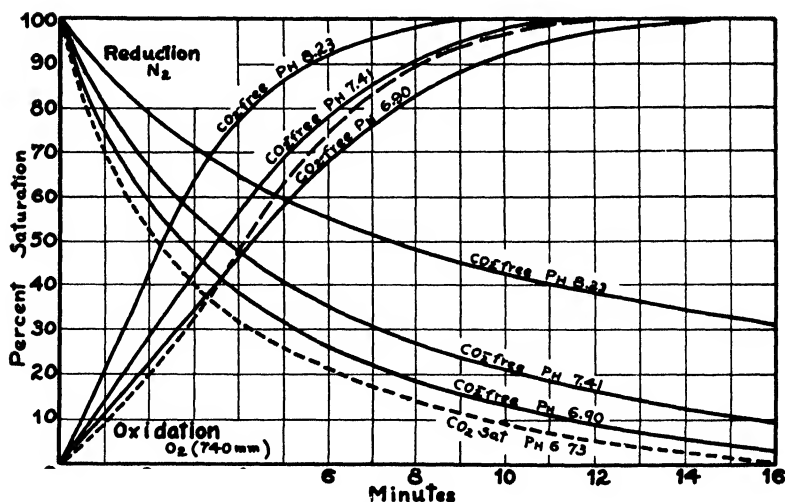


CHART 2

The same blood was then put into the comparing cup and the depth would be recorded on the drum as also 4 mm. However, as

soon as the cup began to revolve, the color density of the blood within increased so that now 3.4 mm. of blood matched the 4 mm. of the standard. This ratio was proportional to the depth so that the revolving depth at any point was about 0.85 that of the resting depth. This correction was taken into consideration in transposing the curves.

The gases used were obtained from compressed tanks. The work was started using hydrogen, but it was found that traces of carbon monoxide sufficient to vitiate the results were present so that nitrogen was used. The nitrogen and CO<sub>2</sub> each contained less than one-half of 1 per cent oxygen. The gas mixtures were made in large carboys and mixed by means of a small rotary pump. The analyses were made by means of a small Haldane apparatus.

The blood used was obtained from the median basilic vein of the author. It was oxalated with the minimum amount of potassium oxalate required to prevent coagulation and immediately

TABLE I.

Blood	CO <sub>2</sub> -free.	CO <sub>2</sub> saturated.
	pH	pH
12 cc.; 3 cc. NaCl 0.8 per cent . . . . .	8 23	6 73
12 " 1.5 cc. NaCl and 1.5 cc. lactic acid . . .	7 41	
12 " 3 cc 0.1 N lactic acid . . . . .	6 90	

chilled in ice water and used within 3 to 4 hours. The blood was equilibrated at 37.5°C. with the gas mixtures at the same CO<sub>2</sub> tension as was afterwards used for the reduction or oxidation. The equilibration was carried on for 20 minutes using five changes of gas.

The curves shown in Chart 1 show the effect of various oxygen tensions on the rate of oxygenation in the absence of CO<sub>2</sub> at 37.5°C. As will be shown later the presence of CO<sub>2</sub> tends to retard the oxygenation at the various tensions to approximately the same degree.

Chart 2 represents the respective rates of reduction and oxidation at various pH values. The continuous lines represent blood samples free of CO<sub>2</sub>. In all these studies 12 cc. of blood were diluted to 15 cc. by either isotonic sodium chloride, lactic acid, sodium bicarbonate, or mixtures of either of the latter with isotonic sodium chloride solution to obtain the desired pH values.



The S-shape of the dotted oxygenation curve is due to the rate of oxygenation being depressed at the beginning due to the high

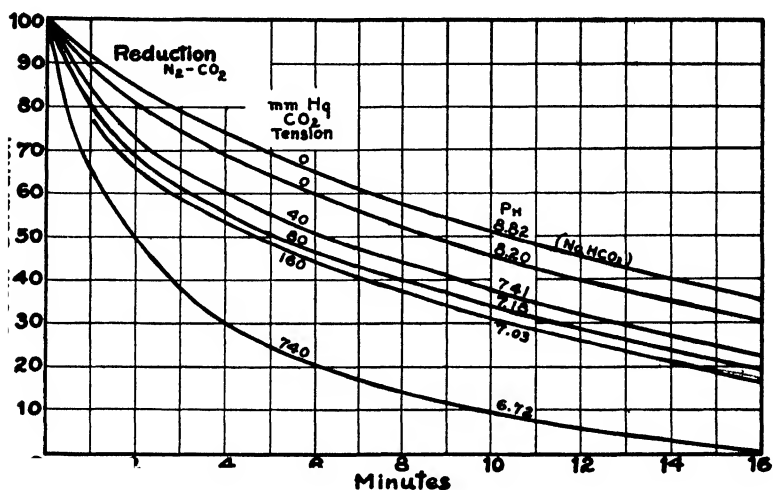


CHART 3

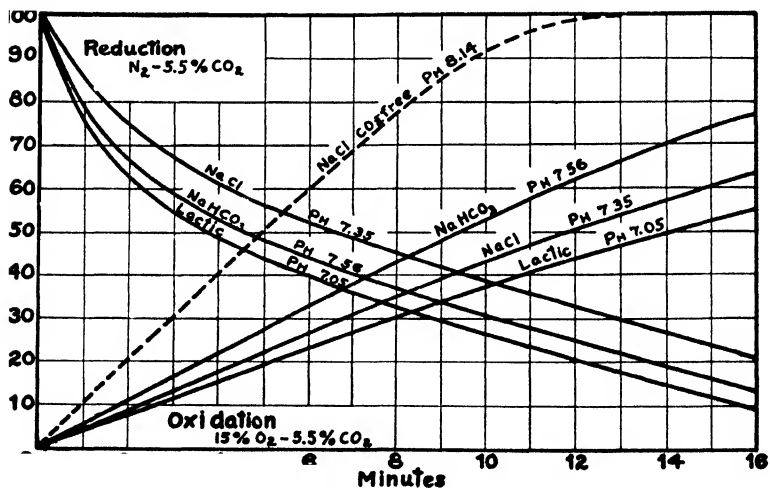


CHART 4.

$CO_2$  tension, but as this is washed out by the pure oxygen the velocity of the reaction increases. The dotted reduction curve represents the velocity when pure  $CO_2$  is used.

Chart 3 represents the rate of reduction as influenced by pH changes brought about by various CO<sub>2</sub> tensions. The blood was first equilibrated with approximately 20 per cent oxygen and the same CO<sub>2</sub> tension as in the nitrogen reduction mixtures except the blood represented by pH 6.72 which was at first equilibrated with 160 mm. (20 per cent) of oxygen and 580 mm. of CO<sub>2</sub> tension.

Chart 4 shows the rate of oxygenation and reduction when the blood remains at a CO<sub>2</sub> percentage of 5.5 per cent. 3 cc. each of saline solution, sodium bicarbonate, and lactic acid were added in each case as before to 12 cc. of blood which were then equilibrated at 37.5°C. with oxygen or nitrogen at a CO<sub>2</sub> tension of 5.5 per cent. The rate of oxidation here is greatly depressed so that it is even less rapid than the rate of reduction. The importance of this phenomenon will be discussed later.

#### DISCUSSION.

The discussion of the oxygen dissociation equilibrium curve has received considerable attention both from the physiologist and the clinician. The thoroughness with which it has been studied has not been applied to the oxygen dissociation velocities, a phase of oxygen transportation which is of high importance. The colorimetric method described lends itself well to the study of this problem. Although the color spectra of reduced and oxygenated blood is different, the color quality as viewed by transmitted light is the same. As a consequence of the spectral difference, variations in the quality of the light effect the depth and quality of the two bloods differently. This necessitates a constant light for all the determinations. The intensity of the light must be great enough to give a penetration of sufficient depth to reduce the error of comparison to a minimum.

The difference in the color depth of blood during rest and rotation is of interest. It is suggested that this difference is due to the orientation of the disk-shaped corpuscles in a plane parallel to the plane of rotation and at right angles to the beam of light. This orientation during rotation permits less light to pass through as compared with their heterogeneous position during rest.

*Oxygen Tension.*

The effect of oxygen tension of inspired or, of still greater importance, alveolar air on the amount of oxygen bound as oxyhemoglobin is well understood. With a normal atmosphere containing 20.9 per cent of oxygen giving an alveolar oxygen content of about 13 per cent the hemoglobin is approximately 95 per cent saturated. A reduction in the oxygen tension of inspired air does not markedly affect the oxyhemoglobin dissociation equilibrium curve until the oxygen percentage has dropped to about 12, or 8 per cent of alveolar oxygen. It is generally accepted that the velocity of the reaction of the combination of oxygen with hemoglobin in the lungs is sufficiently rapid to warrant its saturation to the limit determined by the alveolar tension. Whether or not this is true under all conditions is a question, however. Certainly the observation with a loss as to an explanation of certain clinical anoxemias warrants a consideration of this question.

The discrepancy between arterial saturation and alveolar oxygen tension has for a long time puzzled physiologists. The accurate work of Meakins and Davies (8) shows the arterial oxygen saturation to be within the limits of 94 to 96 per cent in healthy persons at rest— from 2 to 3 per cent less than the alveolar oxygen content. Haldane (9) discusses this problem at length and attributes this difference to defective distribution of the air in the lung alveoli so that the blood in the pulmonary return to the heart is a mixture of blood of varying degrees of saturation. This explanation is an appealing one under normal conditions when the difference is one of a few per cent, but is difficult to conceive when a difference of 50 per cent or more exists without any pulmonary or circulatory involvement. This condition we observed and is the substance of a paper now in preparation showing that in low oxygen tensions with an alveolar oxygen content of 4 per cent, warranting a hemoglobin saturation of 50 per cent, the found saturation of the arterial blood (femoral) was only 16 per cent. This we have repeatedly observed in our anoxemic studies. Haldane (9) summarizes the work on muscular or exertion anoxemia and also reports a difference considerably greater than normal. Here he attributes the deficit due to insufficient time for the required quantity of oxygen to pass through the alveolar epithelium. In

this connection it is also of interest to mention that the arterial saturations of the blood can readily be increased to 100 per cent by breathing pure or nearly pure oxygen.

It is very difficult to say at the present time just what the factors are in incomplete oxygenation of the blood under these conditions. Surely, however, the marked effect that variations in oxygen tension have upon the rate of oxidation as best seen in Chart 1 makes this a factor to be considered. In fact, the static consideration of the saturation curve for hemoglobin falls out of consideration from a practical standpoint inasmuch as arterial blood is never saturated to a degree that alveolar oxygen would permit (except perhaps when pure oxygen is inspired).

The fundamental question, however, concerns the relation of the rate of oxidation or reduction of the method described to the velocities within the body. In other words, what is the relation between the two in surface exposed per unit of time. In the method described 2 cc. of blood stirred at the rate of 165 to 170 revolutions per minute were exposed to a surface of 1,500 sq. mm. That this in no way approximates the surface exposed in the lungs is shown by the fact that minutes were required for saturation rather than a fraction of a second. However, the surface and rate of change show that the velocity of the reaction is not such an instantaneous one as is often supposed.

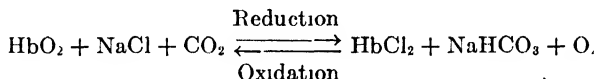
#### *Relation of Reaction to Rate.*

The rôle that CO<sub>2</sub> plays in the various phases of respiration has been a most confusing one. A discussion of the difficult question concerning the specificity of CO<sub>2</sub> will not be attempted in this paper, but evidence will be presented that in certain of the respiratory reactions the specificity rôle of CO<sub>2</sub> must be abandoned and that its action must be attributed to the change in pH that it produces.

The quantitative relationship that CO<sub>2</sub> plays in the oxygen transportation by blood was best expressed by Henderson (10) in his physicochemical system. The definite relationship of CO<sub>2</sub> to pH as determining the oxygen saturation he expressed in the interdependence of six variables as determined by any two.

$$\frac{\text{H}_2\text{CO}_3}{\text{NaHCO}_3} = K \frac{\text{O}_2}{\text{HbO}_2} = K \frac{\text{pH}}{\text{Plasma Cl}}$$

Thus an increase in  $\text{CO}_2$  tension ( $\text{H}_2\text{CO}_3$ ) with a corresponding increase in pH at any definite oxygen tension ( $\text{O}_2$ ) will decrease the amount of oxygen held as oxyhemoglobin ( $\text{HbO}_2$ ). The mechanism of these changes was shown in a graphical fashion by the author in a previous publication (11) and can be shown in the form of an equation without regard to quantities involved as follows:



This equation presents itself as a simple expression upon which both the static and dynamic phenomena of the oxidation and reduction of hemoglobin can be explained. Thus in dealing with  $\text{CO}_2$  on the left-hand side of the equation as a variable, it is evident according to the mass law that for any given tension of oxygen ( $\text{O}_2$ ) the equilibrium obtained and hence the degree of saturation of the hemoglobin with oxygen depend upon the  $\text{CO}_2$  tension present. In a similar manner, the velocity of the reaction up to the point of equilibrium will also depend upon the free  $\text{CO}_2$  content. This is shown in Charts 2 and 3 where an increase in  $\text{CO}_2$  tension increases the rate of reduction and decreases the rate of oxidation as an interpretation of the equation would warrant. Chart 3 further shows that the increase in the rate of reduction as brought about by increased  $\text{CO}_2$  tension is proportional in general to the resulting change in pH. This is further exemplified by the reduction curves on Chart 2 where the rate at pH 6.90 in blood, free of  $\text{CO}_2$  but with lactic acid present, was comparable to the rate of the same blood nearly saturated with  $\text{CO}_2$  and at a pH of 6.73. This is, therefore, a demonstration of a second reaction where accurate methods have been used to show that  $\text{CO}_2$  acts in changing the hydrogen ion concentration.

A third example of a similar nature was demonstrated by Parsons (6). In his study on the change in hydrogen ion concentration in oxygenated and reduced blood, he showed that the change in  $\text{CO}_2$  content of the blood in the respiratory cycle was of the same magnitude as that warranted by the difference that he found in the pH brought about by the concomitant change in the degree of oxygenation.

That the effect of  $\text{CO}_2$  in increasing the rate of reduction and decreasing the rate of oxidation can be duplicated by changing the pH to the same degree by the addition of other acids is shown on Chart 4. It is apparent, therefore, that the effect of  $\text{CO}_2$  or lactic acid is one of hydrogen ion concentration. It must be understood, however, that although hydrogen ion concentration has a specific effect on both the equilibrium and velocity of the oxidation and reduction reactions, that variations in other constituents such as salt concentration may have a marked effect. Barcroft and his pupils (12) first showed the influence of salts present on the dissociation equilibrium curve both in red corpuscles and oxyhemoglobin solution. We have not had the opportunity as yet to study the effect of salt concentration on the velocity curves except in the queer behavior of  $\text{NaHCO}_3$  in the presence of free  $\text{CO}_2$  (5.5 per cent) on the reduction curve as shown on Chart 4. This was a source of great perplexity and made several repetitions of the determinations necessary. Here, in spite of an increase in pH over normal, the rate of reduction is increased. We are at a loss as to an interpretation except attributing it to the specific effect of the sodium ion. In all the bloods where solutions were added (12 cc. of blood and 3 cc. of isotonic solution) an attempt was made to keep the tonicity unchanged and the volume the same. In the case of  $\text{NaHCO}_3$  this is difficult, however, for the change of  $\text{CO}_2$  tension during equilibration changes the amount of the former present and accordingly the osmotic pressure.

No attempt will be made at the present time to discuss the many factors which may play an important rôle in oxygen exchange, such as rate of diffusion through alveolar epithelium, plasma, or corpuscular membrane. The problem dealt with here has been one of the direct exposure of whole blood.

The real question involved in this problem, however, is of what value are these studies in understanding the complex problem of the physiology of respiration. It is hoped that a continued study of the fundamentals involved will enable the clinician to deal more successfully with these problems at the bedside.

The author wishes to take this opportunity to express his appreciation for the kindness shown and the helpful suggestions made by Prof. H. C. Bradley in whose laboratories the work of the past 2 years has been done.

## CONCLUSIONS.

1. A colorimetric method has been described by means of which the rate of oxidation and reduction of blood can be determined with fair physical constancy.

2. Such studies show that oxygen tension markedly affects the velocity of the reactions. It is suggested that this may be an important factor in the anoxic type of anoxemia.

3. An increase in pH increases the rate of oxidation and depresses the rate of reduction. Conversely, a decrease in pH decreases the rate of oxidation and increases the rate of reduction.

4. CO<sub>2</sub> affects the velocities by the pH changes that it produces. Other examples of the non-specificity of CO<sub>2</sub> are cited.

5. Specific ions such as Na may modify to a considerable extent the reduction and oxidation curves.

6. An attempt is made to explain the results obtained on the reduction-oxidation equations presented.

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# THE PRESENCE OF VERNINE (GUANOSINE) IN THE GREEN LEAVES AND BERRIES OF THE COFFEE TREE (*COFFEA ARABICA* L.) AND ITS RELATION TO THE ORIGIN OF CAFFEINE IN THIS PLANT.\*

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After studying the chemical composition of berries of the coffee tree in varying stages of development, I was able to verify that while yet green, besides caffeine, they contain adenine, hypoxanthine, xanthine, and vernine (guanosine), this last in relatively large quantities.

Schulze and coworkers (1) were the first to find this pentoside in young plants of *Vicia sativa*, *Lupinus albus*, and *Trifolium pratense*; in the ripe seed of *Lupinus luteus* and *Arachis hypogaea*; in small etiolated plants of *Cucurbita pepo*; in the green seed of *Pisum sativum*; and in the pollen grains of *Corylus avellana* and *Pinus sylvestris*.

Von Lippmann (2) and Andrlík (3) found this same substance in molasses.

Schulze and coworkers (1) say that the quantity of vernine was always very small and was frequently even not met in plants from which it had already been isolated as in the case with young plants of *Vicia sativa*.

Later, he and Trier (4) found that vernine was identical to the guanosine isolated from nucleic acid by Levene and Jacobs (5).

The discovery of this substance in green leaves and berries of coffee goes further to prove one of the hypothesis as to the origin of caffeine in plants, the hypothesis advocated by Clautriau, Suzuki, Hartwich, Du Pasquier (6), and Pictet and by which caffeine is held to be a product of the decomposition of nuclein in

\* Translated by Carlos de Barros Wright (Agronomo).



precisely the same manner as happens with xanthine in the animal organism.

When searching for caffeine and the alloxuric bases in leaves killed by frost and in green leaves gathered from the same branch, I found that the green leaves contained less nitrogen of the alloxuric bases and of caffeine than did the leaves killed by frost, as is shown by Table I.

The alloxuric bases of the leaves killed by frost were constituted almost exclusively of guanine while in the green leaves as well as in the green berries I found adenine, hypoxanthine, and xanthine in very small quantities; but never free guanine.

As is well known, cell membranes of leaves killed by frost lose their impermeability due to the transformations suffered by the colloids of which they are constituted. The enzymes, which exist in the tissues, now being able to pass through them, reach on the guanosine and are thus the cause of the increased quantities of alloxuric bases found in the coffee leaves killed by frost.

TABLE I

Total nitrogen 100	Green leaves	Leaves killed by frost (7).
Caffeine nitrogen ..	6 79	7 09
Alloxuric bases nitrogen . .	0 45	0 88

It is my opinion that the increased quantity of caffeine, which was observed, is also related to this same phenomenon and that the formation of caffeine in the leaves and berries of the coffee tree is brought about by the transformation of guanosine to guanine, of this latter to xanthine, and lastly, of xanthine to caffeine, due to the action of the enzymes.

#### EXPERIMENTAL.

1,200 gm. of very fine dust of berries which fell in November<sup>1</sup> were extracted several times by water, heated 1½ hours on the water bath, and the proteins, organic acids, tannin, etc., precipitated by neutral acetate of lead, care being taken not to use the reaction in excess.

After having precipitated the excess of lead by H<sub>2</sub>S, the filtrate was concentrated under a reduced pressure of about 500 cc. and

<sup>1</sup> 200 berries weighed 3,217 gm. and contained 69 per cent of moisture.

the caffeine extracted by chloroform until a drop of the chloroform extract no longer left a residue to caffeine. The extract, free of caffeine, was treated by nitrate of mercury according to the method of Schulze and Trier (4).

The precipitate, thus obtained, was filtered, thoroughly washed with cold water, pressed between filter paper, suspended in water, and decomposed with sulfurated hydrogen. The filtrate from mercury sulfide, after being discolored by animal charcoal, was made faintly alkaline to litmus and evaporated under reduced pressure to a small volume, care being taken constantly to verify the reaction of the liquid during the evaporation, in order to keep it always neutral or slightly alkaline.

The solution, after being thoroughly concentrated, formed a jelly, which, on being again dissolved, formed crystals. These crystals were separated by filtration and purified by successive recrystallizations. Thus purified, they formed delicate needles, which, being dried between filter paper, acquired a silky glitter.

They were insoluble in cold water and alcohol, but dissolved readily in boiling water, diluted mineral acids, and alkalis.

The solution of the crystals was precipitated by phosphotungstic acid and nitrate of mercury.

The phloroglucin-hydrochloric acid test was positive and the colored solution thus obtained, extracted by amyl alcohol when viewed before the spectroscope, was found to show a sharp black absorption band in the yellow of the spectrum between the D and E lines.

A solution of the substance in 1 per cent sulfuric acid, heated in an autoclave under a pressure of one and a half atmospheres during 2 hours, on being evaporated to a small volume, formed crystals which, by their characteristic form and by the loss of water of crystallization, on being heated proved to be sulfate of guanine. All the guanine in this solution was precipitated by ammonia and the filtrate treated by an excess of carbonate of barium and evaporated until thoroughly dry. Redissolved in cold water and filtered, the liquid thus obtained was tested for and found to contain pentoses.

3 kilos of leaves gathered in June, containing 63.18 per cent of moisture, treated by the same process, were found to contain guanosine also.

## CONCLUSION.

In the green leaves and berries of the coffee tree of the species "arabica" there exists a pentoside containing guanine. This is probably the guanosine discovered in several plants by Schulze, and by Levene and Jacobs in nucleic acid.

This pentoside is probably the origin of the caffeine in the green leaves and berries of the coffee tree. It is transformed by enzymes to guanine, then to xanthine, and finally from xanthine to caffeine.

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# ON THE EFFECT OF HEMATOPORPHYRIN ON THE DEPOSITION OF CALCIUM IN THE BONES OF RACHITIC RATS.\*

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PLATES 3 to 5.

(Received for publication, November 5, 1923 )

Like that of a photographic plate, the sensitiveness of living cells and organisms to rays of light that lie within the bounds of the visible part of the spectrum can be considerably increased. Raab (1) observed this sensitizing influence of acridine on paramecium and von Tappeiner and Jodlbauer (2) and others found that only certain fluorescent substances possessed this remarkable quality.

Several of these sensitize only *in vitro*, while a few, namely eosin, chlorophyll, and certain derivatives of blood pigment, have also proved effective *in vivo*. With one of these latter, hematoporphyrin, interesting experiments have been made by Hausmann (3), Meyer-Betz (4), and others, which have shown that the sensitizing power of this substance is very strong, though its degree of fluorescence, if compared with that of eosin for instance, is not very high.

Hausmann found that white mice that can stand a dose of 10 mg. of hematoporphyrin without apparent harm, provided they are kept in the dark, are killed within a few hours by a dose of but 2 mg. with symptoms of hyperemia of the skin, itch, activity, dyspnea, and convulsions, if they are exposed to direct sunlight. Less intense light caused chronic poisoning and death after some days, or at least long oversensitiveness to light.

Meyer-Betz injected a solution of 200 mg. of hematoporphyrin into his own blood. Subsequent exposure to sunlight produced most distressing symptoms. On the spot where the injection had been made under the

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\* Dedicated to Professor Dr. C. Eijkman, on the occasion of the 25th anniversary of his appointment as Professor of Hygiene.

influence of Finsen light an infiltration developed, which passed into necrosis. Furthermore, his skin became hypersensitive to sunbeams. A short exposure of his hands to sunlight immediately produced a sensation of burning and after some time strong hyperemia and edema of the skin. His face became so swollen that he could not open his eyes, and it was several days before these symptoms had disappeared. The sensitiveness of the skin to light lasted for a long time.

In connection with these facts it may be remembered that Ehrmann (5) ascribes the idiosyncrasy with regard to sunlight of the so called hydroa vacciniforme to photodynamic action of hematoporphyrin. Mackey and Garrod (6) and others have stated that they have found hematoporphyrin in the urine of sufferers from this malady, which usually occurs in spring. Perutz (7) saw vesicles appear on the skin of rabbits poisoned with sulfonal, which poisoning, as is well known, may be accompanied by hematoporphyrinuria if exposed to rays of the quartz mercury arc

While I was employed on an investigation of the therapeutics of rickets, more in particular on the effect of light, by means of which investigators such as Hess and Unger (8) and Huldshinsky (9) have obtained such remarkable results, I asked myself if hematoporphyrin, which has proved to act as a strong sensitizer, had perhaps any effect on the calcium metabolism of rachitic rats. To ascertain this I subjected some rats suffering from experimental rickets to treatment with this derivative of blood pigment.

Groups of young rats, each group from the same litter, were first fed upon Diet 3143 of McCollum and coworkers (10). This diet is made up as follows:

Ground wheat kernel . . .	<i>parts</i> 33
“ maize “ . . .	33
“ gelatin . . .	15
Wheat gluten . . .	15
NaCl . . .	1
CaCO <sub>3</sub> . . .	3
	---
Total . . .	100

As is well known, and as in this case, roentgenologic and histologic examinations of control animals proved that this diet causes, within a few weeks, changes in the tissues of the skeleton, which in detail show analogy to rickets; as for instance, thickening of the joints, softness of the long bones, curving of the ribs at an angle (rosary), and in connection with this, deformation of the thorax.

The x-ray photographs of the joints show a broad, vaguely and irregularly outlined zone between epiphysis and diaphysis, which points to a defective calcification of the cartilage. This uncommonly wide zone, which is not seen in normal individuals or rachitic rats treated with active light or with cod liver oil if put under the magnifying glass, proves to consist of cartilaginous tissue, in which either no calcium at all or only a small quantity has here and there been deposited in the matrix of the proliferative cartilage. On the other hand, blood vessels have in several places penetrated into the proliferating cartilage. All this has caused a complete loss of the regular structure of the cartilage with its various layers of proliferous, hypertrophic, and preparatory calcified cells. The wall of the long bones is flexible and so soft that it can easily be cut without previous decalcification. It consists for the greater part of osteoid tissue of which only the inner layers are calcified and these incompletely, for they are perforated in various places. The trabeculae of the spongiosa are few, small, and irregular in form and they are embedded in an excess of osteoid. The marrow, on the other hand, is abundant, highly vascularized, and often very fat.

As soon as the animals showed signs of rickets and the morbid changes in the bone tissue of control animals had been established by means of x-rays and the microscope, the former received several times, at intervals of a few days, an injection of 3 to 10 mg. of hematoporphyrin hydrochloride in an alkaline solution; after which treatment they were first examined by x-rays and then killed for the purpose of microscopic examination.

In a number of cases, for the sake of a more reliable control, I set to work in a different way. Before administering hematoporphyrin I amputated one of the hind legs, the long bones of which were then examined in the usual way as the control of the other leg, which was examined in its turn after the treatment with hematoporphyrin had taken place. With a single exception the animals bore the operation, which took place *lege artis* and barely lasted 10 minutes, very well. The healing of the wound proceeded, with a single exception, *per primam* and was so rapid that within a week treatment with hematoporphyrin could be started.

Though it was not likely that the operation *per se* would influence the deposition of lime, I compared, to make sure, the amputated leg of a rat with the remaining one after the animal's recovery, but I did not find any difference between them with regard to the deposition of lime in the bone tissue.

To test the influence of hematoporphyrin on the deposition of calcium in the metaphysis I made use of the line test recommended by McCollum and coworkers (11). Extremely small deposits of calcium in the rachitic metaphysis are clearly observable under the microscope, especially if one uses Kossa's silver test. The sections can then be colored in the usual way; I applied, by preference, van Gieson's stain, because by this the osteoid tissue assumes a bright garnet red and thus forms a striking contrast with the other tissue elements.

When applying the line test one has to take care that the rachitic diet is not continued longer than is necessary to develop rickets; if it is continued too long the process enters the stage of recovery which as we know from clinical experience also comes without therapeutic measures and during which deposition of calcium takes place, which might be misleading.

Besides the histologic examination I also made chemical determinations of the calcium content in some of the long bones. Unfavorable circumstances prevented me from completing this part of the work. Though hoping to find an opportunity to finish this work, I will now observe that I doubt if the results will reveal great differences between the calcium content in the bones of rachitic animals and of those treated with hematoporphyrin. The line test becomes positive with very minute quantities of calcium. It is very doubtful whether the calcium increment can be demonstrated analytically, by weight, as well as by roentgenologic and microscopic means.

For want of better facilities I was obliged to keep the rats in the basement of my own house, into which no sunbeam can penetrate. Furthermore, the sun shone but seldom in Holland during the winter months of 1922-1923 and the diffuse daylight was for the greater part intercepted by adjacent houses. As a matter of fact the bad light in the room in which both the control rats and those treated with hematoporphyrin were kept (both under exactly the same conditions) was of little importance as the window glass absorbs all the rays that effect rickets. For rays of shorter wavelengths than  $334\text{ m}\mu$  are not transmitted by this kind of glass, at least not by the kind that Hess and Weinstock (12) used, and it is rays the wave-length of which is not above  $312\text{ m}\mu$  that have a therapeutic effect on rachitis. This explains why, with a suitable

diet, rachitis can always be effected, even in rooms where the light is better than in mine.

It also appeared from my experiments that hematoporphyrin is by no means a harmless substance. Though the animals were not exposed to sunlight, they bore the injections very badly. Some animals died the very next day and others lost weight during the treatment, which must probably be partly ascribed to their loss of appetite. Two rats died the day after the injection of 10 mg.; another was extremely sick after a dose of 9 mg., so that I considered it best to kill it. A white rat lost 10 gm. after two doses of 8 mg. in a week; another lost 32 gm. in consequence of 5 mg. three times in 7 days.

The influence of hematoporphyrin on the deposition of calcium in the metaphysis was unmistakable. In all cases the line test was positive and in most cases the honeycomb-like deposit was much broader than the minute line of calcium that McCollum speaks of, so that the microscopic picture showed a striking resemblance to that of animals treated with cod liver oil or with irradiation. In accordance with this the bones of the animals after treatment with hematoporphyrin proved to be harder and were more difficult to cut for the sections.

The difference in the x-ray photographs is also striking. Instead of the broad, vaguely and irregularly defined zone between epiphysis and diaphysis of the rachitic animals, there is to be seen on the plates of those treated with hematoporphyrin only a narrow, clear, and regular strip, which, in perfect agreement with the microscopic picture, betrays an increased deposition of lime in the metaphysis.

Hence the therapeutic effect of hematoporphyrin in experimental rickets of the rat cannot be doubted. The question now arises how this effect can be explained. Is it direct or indirect?

On the ground of what is known concerning the photosensitizing quality of hematoporphyrin the latter is more probable. It is very likely that this substance causes great sensitivity to visible rays of light, the photodynamic effect of which is but small in comparison with the ultra-violet rays and is entirely inefficient with regard to rachitis. If this supposition is admissible there is some reason to assume that the sensitizing power of hematoporphyrin is very strong, for as was said before, the room where



the animals were housed was badly lighted. The question, however, can only be solved if the experiment with hematoporphyrin is conducted in such a way that the admission of light is quite prevented, as in absolute darkness. I was unable at the time to make the experiment in this way. I did, indeed, try to make this necessary control possible by shutting up some of the animals in a cupboard, but it was not quite light-tight and besides, it had to be opened repeatedly for cleaning the cages and feeding the animals, and the injections had to be done in daylight. The animals thus treated reacted positively to the hematoporphyrin. However, I have now found an opportunity for experimenting in absolute darkness and hope to communicate the results in due time.

It is a pleasure to acknowledge my indebtedness to Professor E. Ringer for the hospitality in his Laboratory of Physiological Chemistry at Utrecht, and to express my thanks to Professor W. Schuffner, of the Institute of Tropical Hygiene at Amsterdam, for his help in making the photomicrographs.

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## EXPLANATION OF PLATES.

## PLATE 3.

FIG. 1. Radiograph of one of the hind legs of normal albino rat No. 131. This animal served as a control and has been kept on the breeding diet. It belongs to the same litter as a group of rats, which has been kept on McCollum's diet No. 3143.

FIG 2. Radiograph of the hind legs of albino rat No 144 It has been kept on McCollum's diet No 3143 and has suffered from rickets Note the enlarged, wavy and indefinite outlined zone of proliferative cartilage of the tibia. Defective calcification. Note also the dislocation of the distal epiphysis of the left femur.

FIG 3 Radiograph of the hind legs of black and white rat No. 199 McCollum's diet No 3143 *Treated with hematoporphyrin.*

Date	Weight	Remarks
	<i>gm</i>	
Oct. 30	52	
Nov. 9	58	Injection of 3 mg. hydrochloride hematoporphyrin.
" 13	58	" " 5 " " "
" 16	54	" " 5 " " "
" 20	51	
" 23	50	
" 27	54	Injection of 5 mg. hydrochloride hematoporphyrin.
" 30	50	
Dec. 4	50	Injection of 5 mg hydrochloride hematoporphyrin.
" 7	46	" " 5 " " "
" 10		Dead

The long bones of the legs are but slightly flexible and are rather hard Note the well defined margins of the metaphysis

FIG. 4. Radiograph of the knee joints of black and white rat No 197, taken post mortem. McCollum's diet No. 3143 *Treated with hematoporphyrin.*

Date.	Weight	Remarks
	<i>gm</i>	
Oct. 30	56	
Nov. 9	62	Injection of 3 mg hydrochloride hematoporphyrin.
" 13	58	" " 5 " " "
" 16	50	" " 5 " " "
" 27	56	" " 5 " " "
Dec. 4	50	" " 5 " " "
" 5		Dead.

The long bones were hard, not flexible. Note the narrow zone between epiphysis and diaphysis. The normal contour of the metaphyseal margins is nearly restored.

FIG. 5. Radiograph of the knee joints of albino rat No. 142, taken post mortem. McCollum's diet No. 3143. *Treated with cod liver oil.*

Date	Weight	Remarks
	<i>gm</i>	
Nov. 9	61	
Dec. 4	81	2 gm. of cod liver oil daily.
" 14	75	Diarrhea
" 18		Dead

*Autopsy* — Meteorism and enteritis. Edema of the tail and the hind legs. Note the resemblance to the radiographs of Rats 199 and 197.

*BM*, bone marrow, *CC*, zone of columns of proliferous cartilage, *CCB*, calcified cortical bone, *Ep*, epiphysis, *FC*, fat cells, *HC*, zone of hypertrophic cartilage cells, *Mct*, metaphysis, *O*, osteoid tissue, *PC*, provisional calcification of proliferous cartilage; *Per*, periosteum, *Sp*, spongiosa.

FIG. 6. Photomicrograph of a section of the distal part of the femur of black and white rat No. 219. Normal control animal. Diet: brown bread, milk, butter, cheese, and cabbage. Partly decalcified in Muller's solution. Staining: silver-van Gieson. Magnification  $\times 15$ . Normal calcification of epiphysis and diaphysis as well as of the proliferous cartilage.

#### PLATE 4

FIGS. 7 and 8. Photomicrographs of the distal parts of the right and left femurs of albino rat No. 137. McCollum's diet No. 3143. *Treated with hematoporphyrin.*

Date	Weight	Remarks
	<i>gm</i>	
Nov. 30	109	
Dec. 11	114	
" 15		The radiograph shows a broad metaphyseal zone.
Feb. 5	148	
" 21		Amputation of the right hind leg
" 26	137	Injection of 8 mg. hydrochloride hematoporphyrin
Mar. 3		" " 8 " " "
" 6	127	Killed with chloroform.

*Autopsy.*—Rosary. Bones not very hard.

FIG. 7. Femur of right hind leg *before* the treatment with hematoporphyrin. Partly decalcified. Silver-van Gieson. Magnification about 12. Defective calcification of epiphysis and diaphysis. Cortical bone thin, consisting for a great part of osteoid tissue. Proportion of osteoid tissue to calcified bone tissue 1:2, or even 1:1. Trabeculae of the spongiosa also thin, here proportion is 1:2. Zone of columns of proliferous cartilage cells enlarged. No preparatory calcification of proliferous cartilage, only here and there some provisional calcification.

FIG. 8. Femur of the left hind leg of the same animal *after* treatment with hematoporphyrin. Partly decalcified. Silver-van Gieson. Magnification about 12. Zone of columns of proliferous cartilage cells much less enlarged than in Fig. 7. *Positive line test.* Distinct deposition of calcium in the proliferative zone of cartilage.

FIGS. 9 and 10. Photomicrographs of the epiphysis of the right and left femurs of black and white rat No. 227. McCollum's diet No. 3143.

Date	Weight	Remarks
	gm	
Feb. 12	62	
Mar. 16	84	Amputation of right hind leg. The long bones of this leg are soft and flexible. While preparing the bone the epiphysis tears off.
" 21	77	Injection of 7 mg. hydrochloride hematoporphyrin.
Apr. 5	78	" " 4 " " "
" 11	74	" " 4 " " "
" 20	70	Killed with chloroform.

*Autopsy.*—Pale, gray colored muscles. Rosary. Bones moderately soft, less soft than those of the amputated leg. In the vesica one brown calculus of 1 mm. diameter.

FIG. 9. Insufficient calcification of epiphysis, better calcification of diaphysis. Cortical thin. Much osteoid. Enlargement of proliferative zone of the cartilage, not regularly outlined by epiphysis and diaphysis. Partial calcification of metaphysis. Irregular ingrowth of blood vessels. Disorganization of zone of columns of proliferous cartilage cells.

FIG. 10. *Line test positive.* For the rest, wall of the bone thin. Much osteoid. Poorly developed spongiosa.

#### PLATE 5

FIGS. 11 and 12. Photomicrographs of the proximal parts of the right and left tibiae of black and white rat No. 231. McCollum's diet No. 3143. Not decalcified. Silver-van Gieson. Magnification about 12.

Date	Weight	Remarks
	<i>gm</i>	
Feb. 13	70	
Mar. 7	82	
" 13		Amputation of right hind leg The long bones soft and flexible Enlargement of the metaphysis.
" 17	80	Injection of 9 mg hydrochloride hematoporphyrin.
" 20	76	" " 9 " " "
" 22	74	" " 9 " " "
" 30	71	Killed with chloroform

*Autopsy*—Pale, gray colored muscles Bones soft. In the vesica some brown concretions, not over 1 mm in diameter

FIG 11. Proximal part of tibia of amputated right hind leg. Important enlargement of zone of proliferous cartilage On two spots only some preparatory calcification Much osteoid

FIG. 12 Proximal part of tibia of left hind leg after treatment with hematoporphyrin *Line test positive* Calcification of the greater part of metaphysis Also beginning of calcification of the peripheral osteoid tissue

FIGS 13 and 14 Photomicrographs of the proximal parts of the tibiae of the right and left hind legs of black and white rat No 229 McCollum's diet No 3143. Not decalcified Silver-van Gieson Magnification about 12

Date	Weight	Remarks
	<i>gm</i>	
Feb 13	61	
" 26	64	
Mar. 7	66	
" 15	66	Amputation of right hind leg Long bones very soft and flexible
" 21	56	Injection of 10 mg hydrochloride hematoporphyrin.
" 22	55	
" 23		Dead.

*Autopsy*—Bones somewhat harder than those of right leg.

FIG 13 Proximal part of the tibia of the amputated leg. Important enlargement of the zone of proliferative cartilage. One-half of this zone consists of cartilage tissue, the other half partly of cartilage, partly of osteoid Between osteoid and cartilage tissue there is an irregular layer of calcium. Much osteoid

FIG 14 Proximal part of the tibia of the left hind leg after treatment with hematoporphyrin. The calcium deposit takes up the whole breadth of the metaphysis. *Positive line test*



(van Iersum Calcium bones of rachitic rats)





(van Iersum: Cu in bones of rachitic rats)









# THE AMINO-ACID CONTENT OF THE BLOOD IN NORMAL AND PATHOLOGIC CONDITIONS.

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The present day concepts of the amino-acid metabolism in normal persons have been formulated largely on the basis of the studies of Folin and Denis, Van Slyke and Meyer, and Folin and Berglund. These authors have been particularly interested in the relation of the amino-acids in the blood to the processes of protein metabolism in normal persons.

The behavior of the amino-acids in the blood in disease has not been investigated so thoroughly. On the basis of his study of normal persons, Folin says: "The deamination process appears to be such a fundamental process that one cannot expect to find many pathological conditions in which the amino nitrogen of the blood filtrates will vary very much from the normal." Various analyses of the blood in disease have been reported, but not in a series sufficiently large to establish this generalization. Furthermore, the results reported have not been sufficiently uniform to be of great value in the study of the deamination process.

The quantitative study of the amino-acids in the blood has been difficult because of the inadequacy of the earlier methods of analysis; indeed it was not until 1914 that Abel, Rowntree, and Turner first isolated crystalline amino-acids from the blood stream by their method of vividiffusion. The present methods of analysis are more satisfactory, but the exact amount of amino nitrogen found in a sample of blood depends largely on the details of the analytic method used. Amino-acids are more concentrated in the blood corpuscles than in the serum.

The method of protein precipitation is important. Hiller and Van Slyke have recently compared the more commonly used reagents, most of which permit similar amounts of amino nitrogen to pass into the blood filtrate. After alcohol precipitation, however, the filtrate contains only about two-thirds as much amino nitrogen as the filtrates after trichloroacetic acid, or tungstic acid precipitation, although the proportion of the amino nitrogen in the alcohol filtrate is fairly constant. This objection to the method of alcohol precipitation in no way affects the validity of the conclusions drawn from comparative physiologic experiments, but the results of Van Slyke and Meyer, and of others using the method cannot be compared directly with analyses of filtrates prepared according to the methods of Bock or of Folin and Wu. The postprandial amino-acidemia must also be considered in interpreting analytic values found in blood samples not taken under conditions of fasting, although Hammett found practically the same amino nitrogen content in blood samples taken from 3 to 4 hours after a meal, as in samples after fasting.

In this study, analyses were made of whole blood from a series of disorders as extensive as possible, with special reference to known disorders of metabolism. All blood samples were taken in the morning, before breakfast. The colorimetric method of Folin was used for determining the amino nitrogen. Duplicate analyses of standard solutions of alanine by the nitrous acid method of Van Slyke gave constant analytic values, although a somewhat more uniform series of readings was obtained by the colorimetric method. Duplicate analyses of a small series of blood filtrates indicated that the results by the Folin method were, on the average, slightly higher (0.5 mg.) than those by the Van Slyke method. Because of the greater convenience, the colorimetric method was used routinely in the present study.

#### DISCUSSION OF RESULTS.

A summary of the analytic data is given in Table I. The average, minimal, and maximal values for the amino nitrogen found in each condition are given. The constancy of the analytic values is striking, and in none of the conditions studied was there a significant deviation from the normal. Certain of these conditions warrant individual discussion.

*Normal.*—In a series of twenty observations on twenty normal persons, the amino nitrogen in the whole blood was found to

TABLE I  
*The Amino-Acid Content of the Blood in Normal and Pathologic Conditions*

Diagnosis	Observations	Amino-acid nitrogen in 100 cc		
		Minimal	Maximal	Average
		mg	mg	mg
Normals	20	5.2	7.2	6.37
Renal insufficiency, blood urea greater than 50 mg in 100 cc	56	4.9	8.6	6.52
Chronic glomerular nephritis	21	5.1	8.6	6.57
Pyelonephritis	17	4.9	8.1	6.55
Hypertension and arteriosclerosis, blood urea normal	20	4.8	7.7	6.51
Myocardial degeneration with cardiac decompensation	20	4.7	8.3	6.83
Exophthalmic goiter, basal metabolic rate above +15	44	4.6	7.7	6.00
Adenoma of the thyroid with hyperthyroidism, basal metabolic rate above +15	27	5.1	8.0	6.31
Myxedema and hypothyroidism, basal metabolic rate below -10	25	4.9	8.2	6.11
Diabetes, blood sugar over 0.2 per cent	86	4.6	8.0	6.06
“ “ “ below 0.2 “ “	30	5.1	7.6	6.31
Obesity	17	5.6	8.0	6.75
Chronic arthritis	11	5.9	8.2	6.67
Carcinoma	19	5.3	7.4	6.22
Hepatic insufficiency as shown by the phenoltetrachlorophthalein test	19	4.7	8.1	6.30
Anemia	6	5.1	8.0	6.38
Tetany	5	5.0	7.2	5.76
Duodenal ulcer, sippy regime	5	5.7	6.7	6.34
Pneumonia	3	5.5	6.6	6.10
Typhoid fever	1			6.50
Addison's disease	3	5.8	6.0	5.90
Acromegaly	1			5.90
Gout	2	6.3	6.8	6.55
Total	458	4.6	8.6	6.32

vary between 5.2 and 7.2 mg. in 100 cc., the average amount being 6.37 mg. This compares favorably with the analyses of

Folin and Berglund. In a series of twelve normal, fasting subjects, they found that the blood contained from 5.7 to 7.8 mg. of amino nitrogen, an average of 6.4 mg. These two series of estimations were made by the same method under similar conditions. The analyses of Bock gave slightly higher amounts, 7.72 mg., while those of Hammett were slightly lower, 4.9 mg. Blau, also using the gasometric method, obtained values for the normal comparable with those of Hammett. The other reported analyses of normal human blood come within the foregoing limits.

Percentage of  
Occurrence

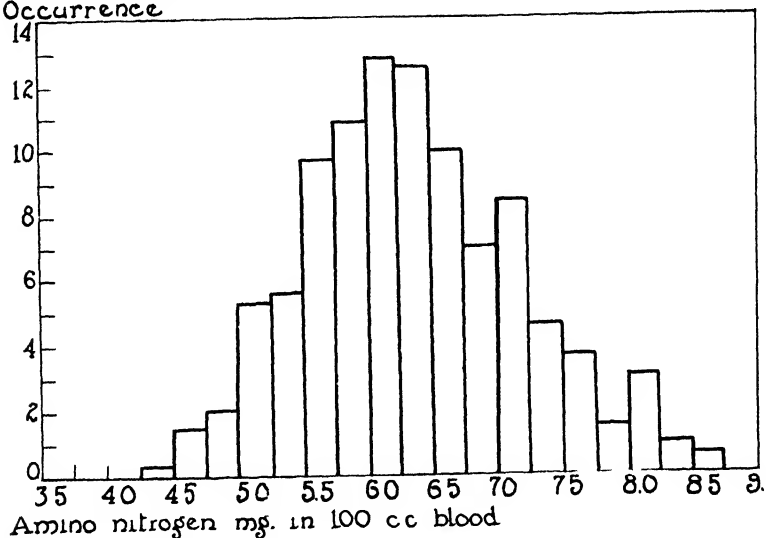


FIG. 1. Frequency curve showing the distribution of different concentrations of amino nitrogen in human blood over the range of occurrence.

Frequency curves were constructed, showing the distribution of amino nitrogen in the blood of normal persons, and that of the entire group studied. As there was no significant divergence between the two series, only the curve for the larger group is shown (Fig. 1). This frequency curve is fairly symmetric. 90 per cent of the observations are between the values of 4.8 and 7.8 mg. The most probable value for the amino nitrogen content of the blood is between 6.0 and 6.4 mg., while the average is 6.3 mg.

*Renal Insufficiency.*—Bock, in particular, emphasized the possibility of an increase in the amino nitrogen in the blood accompanying the retention of other forms of nitrogen in cases of chronic nephritis and uremia. Okada and Hayashi have also reported such an increase in dogs, following ligation of both ureters. Folin and Berglund, on the other hand, did not find evidence of amino-acid retention in a small series of patients with high blood urea. In our study, the observations were classified according

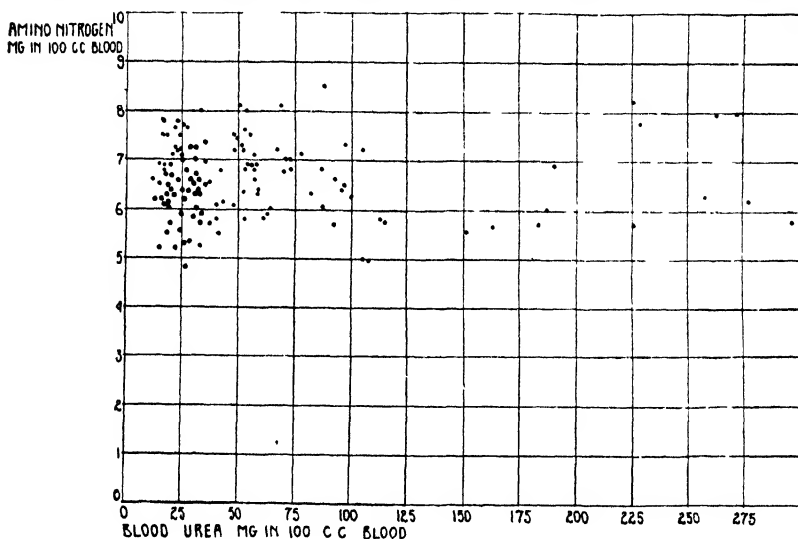


FIG 2. The amino nitrogen in the blood compared with the blood urea in patients with renal insufficiency.

to the clinical diagnosis. Also, all cases in which the blood urea was more than 50 mg. were grouped. The relation between the amino nitrogen and the blood urea is shown in Fig. 2. There is no significant departure from normal in any of the foregoing groups, and no apparent correlation between the amino-acids and the degree of renal insufficiency or urea retention.

The similarity between the results obtained by the colorimetric and gasometric methods of analyzing the blood of normal persons has been mentioned. Urea reacts slowly with nitrous acid, and, if present in moderate amounts, will lead to high readings



by the Van Slyke method unless appropriate corrections are made. Comparison between the two methods when tested against a known alanine solution and the solution with 300 mg. of urea added in 100 cc. is shown in Table II. The colorimetric method gives similar analytic values in the different solutions, and the accuracy is not affected by the excess of urea. No attempt was made to remove the urea by treatment with urease, but the usual corrections were made in the gasometric analysis. Nevertheless,

TABLE II

*Comparison between the Folin and Van Slyke Methods for Determining Amino Nitrogen.*

	Alanine solution		Alanine solution with 300 mg urea in 100 cc		Alanine solution with 300 mg glucose in 100 cc
	Folin	Van Slyke	Folin	Van Slyke	Folin
	mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc
	8 25	8 75	8 49	12 20	8 59
	8 58	8 65	8 69	11 10	8 70
	8 26	8 20	8 27	9 40	8 72
	8 45	8 34	8 23	11 80	8 54
	8 44	8 47	8 92	11 50	8 68
	8 59	8 20			8 76
	8 42	8 61			8 68
	8 54				8 80
Average	8 45	8 45	8 50	11 40	8 70
“ deviation	±0.15	±0 20	±0 20	±0 50	±0 10

the gasometric method shows a relatively large error as a result of the added urea, and the possibility of such error must be considered in interpreting previous analyses by that method.

We have repeated the experiments of Okada and Hayashi, but were unable to confirm their finding of an increase in the amino-acids in the blood following nephrectomy. The changes in the blood urea and in the amino nitrogen in a dog, following bilateral nephrectomy, are shown in Fig. 3. The increase in the blood urea apparently is directly proportional to the length of time after operation. The amino-acids, on the other hand, show no change during life. Blood taken post mortem contained nearly double the previous amount of amino nitrogen. Whether this

increase is strictly post mortem or develops during the last 12 hours of life we are not prepared to say. In any event it is essentially a terminal condition. Haden and Orr found no change in the amino nitrogen content of the blood following intestinal obstruction, although there was a marked increase in the blood urea, with accompanying evidence of protein destruction.

*Thyroid Disorders.*—The metabolic changes in diseases of the thyroid gland are well recognized. The metabolic rate is an accurate index of the degree of the disturbance, especially in cases of exophthalmic goiter. However, no correlation was found

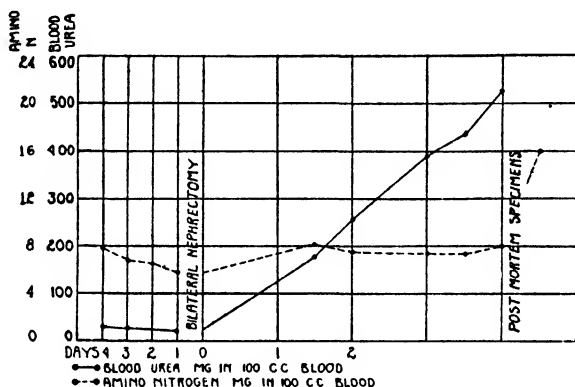


FIG 3. Changes in the amino nitrogen and the urea content of the blood of a dog following bilateral nephrectomy. The increase in the amino nitrogen in the blood obtained after death is shown.

between the amino nitrogen in the blood and the metabolic rate (Fig. 4). The amino-acid content of the blood does not differ from the normal in cases of hyperthyroidism or hypothyroidism.

*Diabetes.*—Desqueyroux has suggested that the amino-acids in the blood are increased in cases of diabetes, especially in obese persons. Neither in diabetes of any type nor in obesity did we find significant changes from the normal. The amino-acids are not correlated with the level of the blood sugar (Fig. 5). This was true both of patients under treatment and of those with diabetic coma and acidosis.

Folin and Berglund noted a slight reduction in the nitrogenous constituents of the blood, including the amino-acids, after the ingestion of 200 gm. of glucose. They said: "As was to be expected from the absence of nitrogen intake and from the protein-

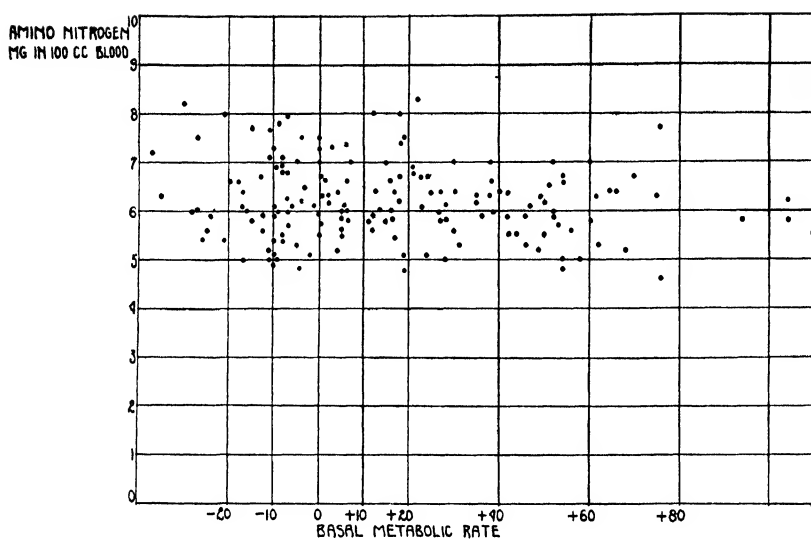


FIG. 4. The amino nitrogen in the blood compared with the basal metabolic rate

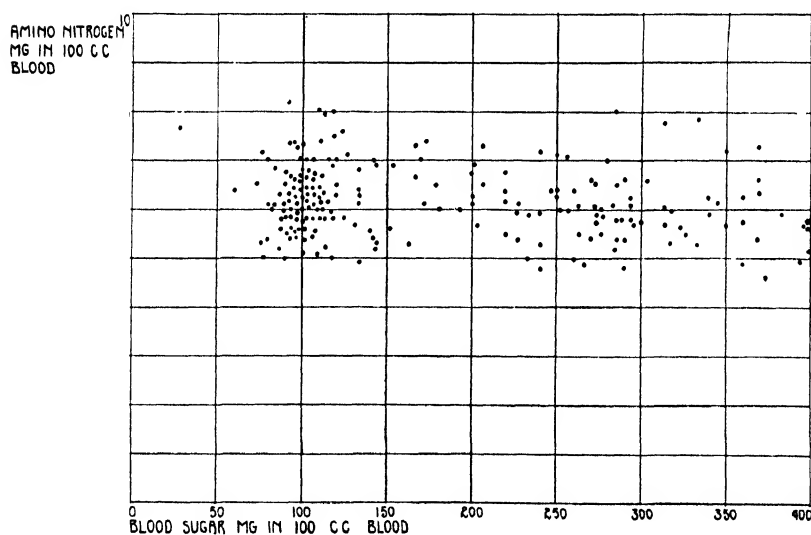


FIG. 5 The amino nitrogen in the blood compared with the blood sugar level.

sparing action of the sugar we meet here with small, but definite reductions in the different nitrogenous constituents in the blood." Through the courtesy of Drs. Wilder and Kitchen, we were able to follow the amino-acids in the blood in their study of the effects of insulin on the utilization of different sugars. There was a slight, though definite, fall in the amino nitrogen after the ingestion of 100 gm. of sugar. This change was entirely similar to that reported by Folin and Berglund, the period of minimal amino nitrogen in general coinciding with the maximal blood sugar levels. There was no change in the hemoglobin during the experiment, showing that the fall in the amino-acids could not be referred to changes in the blood volume or in the relative proportions of plasma and corpuscles present. Levulose produced less change than did glucose. Insulin had no effect on the amino-acids. The degree of fall in the amino nitrogen bore no apparent relationship to the respiratory quotient or to the increase in metabolism accompanying the oxidation of the sugar. We do not believe that the relation between the sugar and the amino-acids is as simple as that postulated by Folin and Berglund, but are unable to account satisfactorily for these changes.

*Hepatic Insufficiency.*—Opinions regarding the effect of hepatic disease on the amino-acids in the blood have varied in proportion to the degree of emphasis placed on the rôle of the liver in amino-acid metabolism. Amino-acid excretion in cases of acute yellow atrophy of the liver may be so increased that leucine and tyrosine crystals appear in the urine. The amino nitrogen in the blood was greatly increased in the cases studied by Feigl and Luce and by Stadie and Van Slyke. In cases of acute yellow atrophy, the hepatic insufficiency is complicated by the rapid autolysis of that organ and the consequent flooding of the system with amino-acids of autolytic origin. An accumulation of amino-acids need not necessarily be ascribed to a failure of deamination and urea formation.

Changes in the blood with acute yellow atrophy alone cannot be used as a standard for determining hepatic insufficiency in other disorders of the liver. Rowntree, Marshall, and Chesney have made the most extensive study of the amino-acids in the blood in hepatic disease. They suggest that the amino nitrogen may be increased in a considerable proportion of such cases. They

used blood serum in their study and also the alcoholic filtrate for analysis; hence the values they obtained for the amino nitrogen cannot be compared directly with the present data. The increases they found were not striking, especially in view of the low value for amino nitrogen taken as the upper limit of normal. Marshall and Rowntree also found a slight increase in the amino-acids in the blood of dogs after phosphorus poisoning. They point out that this increase is largely terminal, and examination of their protocols shows that the highest values were obtained in postmortem samples of blood.

In this study an attempt was made to confirm the degree of hepatic insufficiency by means of the Rosenthal modification of the phenoltetrachlorophthalein test of Rowntree, Hurwitz, and Bloomfield. This test is not an absolute gauge of hepatic function, but in conjunction with the accompanying clinical findings it affords a convenient index of severe functional disturbance. In all the cases reported, largely cirrhosis or chronic hepatitis, there was marked retention of dye in the blood plasma 2 hours after injection. No evidence of disturbance in the amino-acid content of the blood was found. Unfortunately, no cases of acute yellow atrophy were available.

*Leucemia.*—Martin and Denis found evidence of an increase in the rest nitrogen in the blood in cases of leucemia. They suggested that this increase might be due to amino-acids, and this suggestion has since been abundantly confirmed by Okada and Hayashi, and by Sandiford, Boothby, and Giffin. The latter found that in leucemia the values for the amino nitrogen in the whole blood ranged between 5.0 and 16.0 mg., with an average value of 10.0 mg.,<sup>1</sup> and were interested in a possible relationship between this finding and the increased metabolism usually occurring in this disease.

The increased excretion of uric acid in cases of leucemia has long been recognized as evidence of a rapid destruction of leucocytes. Collip, in particular, has emphasized the large amount of amino-acids present in the cell nucleus. It seems plausible, therefore, to ascribe the high amino-acid content of the blood in leucemia to the increase in the leucocytes and an associated flooding of the organism with amino-acids of autolytic origin.

<sup>1</sup> A detailed study of the metabolism in leucemia is in progress.

*Miscellaneous*.—In the other conditions, such as carcinoma, gout, and febrile diseases (Table I), noteworthy changes in the amino-acid content of the blood are not shown, and an examination of the literature did not indicate that changes were to be expected.

#### CONCLUSIONS.

The amount of amino nitrogen in the blood varies between 4.8 and 7.8 mg. in 100 cc., the average amount being 6.3 mg. This was found to be true in normal persons, and in a series of more than 400 observations covering twenty pathologic conditions. The level of amino nitrogen may be increased by flooding the organism with amino-acids arising during digestion or from the rapid autolysis of body tissue, as has been noted in cases of leucemia and acute yellow atrophy of the liver. In general, the quantity in the blood is maintained within the foregoing limits with remarkable constancy. Such disease conditions as uremia, diabetes, exophthalmic goiter, or hepatic insufficiency are not exceptions to this rule.

The observed constancy of this regulation in the presence of such severe metabolic disturbances is direct evidence of the widespread and fundamental nature of the deamination processes in the body.

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## LYSOLECITHINS AND LYSOCEPHALINS.

### II. ISOLATION AND PROPERTIES OF LYSOLECITHINS AND LYSOCEPHALINS.

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In a previous publication,<sup>1</sup> Levene and Rolf showed that by the action of cobra venom on egg yolk a product is formed which consists of a mixture of lysolecithin and lysocephalin. The evidence available at the time was to a certain extent of indirect character. In order to furnish more conclusive proof of this assumption it was necessary to isolate the components. This isolation is now accomplished. In several other respects also, the preparation of lysolecithin and lysocephalin was important. To begin with, the physical properties, especially the solubilities of these substances, are such as to permit a fairly accurate determination of their dissociation constants. The latter ought to be practically the same for the lyso derivatives as for the parent substances. It is possible that a rational method of separation based on the dissociation constants might be devised for the lyso derivatives, and then successfully applied to the separation of cephalin from lecithin. Up to date, the latter problem has not yet been solved. The purest sample of unchanged cephalin contained 25 per cent of lecithin. Free from lecithin were prepared only such samples of cephalin as contained a large proportion of lysocephalin.

Further, lyso derivatives present a convenient material for the investigation of the nature of the saturated fatty acids occurring in the molecules of lecithin and cephalin.

Finally, these substances may serve as a convenient material for the preparation of pure synthetic lecithin and cephalin.

<sup>1</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1923, *lv*, 743.

Before referring to the method of separation and to the properties of these two substances, a few words may be said regarding the preparation of the material.

It was noted early in the work that the addition of large quantities of antiseptic to the digestion mixtures affected the yield very unfavorably. On the other hand, complete absence of antiseptic did not give satisfactory results. Best results were obtained when antiseptic was added, in sufficient quantity to retard the putrefactive processes, but not enough to prevent bacterial growth entirely. Thus it seemed that bacterial growth might have played a considerable part in the production of lysolecithin and lysocephalin. The fact, however, that these substances are produced within 2 hours after digestion has begun, and the fact that the largest yield was obtained in an experiment lasting only 6 hours, seem to indicate that the process of formation of the lyso-derivative is principally of an enzymatic nature.

The details of separation of lysocephalin from lysolecithin are given in the experimental part. Here it is sufficient to state that the mixed two substances are precipitated by cadmium chloride. As with the dihydro derivatives of cephalin and lecithin, so in the case of the lyso derivatives it was found that lysocephalin is the more insoluble in organic solvents and could be purified by crystallization from a solution in chloroform. Lysocephalin crystallizes in beautiful transparent needles which soften at 140°C. and melt with decomposition at 198°C. The specific rotation in glacial acetic acid was +2.0°. On hydrolysis of lysocephalin only one acid, namely stearic, could be isolated. Thus, the older observations on the saturated fatty acids of cephalin<sup>2</sup> are corroborated. Lysolecithin is very much more soluble than lysocephalin and when nearly pure, may be recrystallized from chloroform, pyridine, and methyl and ethyl alcohols. The substance crystallizes in aggregates of needles. It has no sharp melting point, but softens at 100°C. and decomposes at 263°C. The specific rotations in chloroform, pyridine, and glacial acetic acid are -2.6, +1.2, and +0.8°, respectively. There is, therefore, a marked difference between the specific rotations of lysocephalin and lysolecithin. On hydrolysis, lysolecithin yielded palmitic

<sup>2</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlv1, 193.

and stearic acids, thus again confirming the results obtained with pure lecithin.<sup>3</sup>

Regarding the dissociation constants, lysolecithin and lysocephalin, as any other amino-acids, were each expected to have two dissociation constants. For lysolecithin,  $K_1 = 0.18$ ,  $K_2 = 1.3 \times 10^{-12}$ , and the isoelectric range is between pH 2.75 and 9.90; for lysocephalin,  $K_1 = 3 \times 10^{-4}$ ,  $K_2 = 3.4 \times 10^{-10}$ , and the isoelectric range is between pH 5.5 and 7.5.

From our present knowledge it is impossible to distinguish which pK is the acid and which the basic one. By analogy with the older conception of amino-acids, the lower one should be the basic and the upper one the acidic. Then at pH values between the pK's, these substances would exist largely as undissociated molecules.

However, Bjerrum<sup>4</sup> considers that the constants for amino-acids are the reverse of the usual conception. If this is true, and the lysolipoids are dissociated in an analogous manner, then the lower pK would be the acidic one and the upper the basic. Hence between them the substances would exist largely as doubly dissociated. Both groups would be simultaneously ionized, producing an internal salt.

The isoelectric range is given in the experimental part. It represents the pH range over which the substances are less than 1 per cent dissociated according to the older conception of amino-acids. If they correspond to Bjerrum's conception, then this value is the pH range over which there is more than 99 per cent dissociation of both groups.

The isoelectric "point" for lysolecithin is 6.33 and for lysocephalin 6.5, but these values have no practical significance owing to the wide range in which there is no buffer effect.

Physiologically, it was of interest to determine whether the hemolytic property of the mixed lyso derivatives was the common property of both derivatives or of only one. The experiments reported here were carried out by Dr. Hideyo Nogouchi and show that both lysolecithin and lysocephalin possess this property.

<sup>3</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, lrv, 92.

<sup>4</sup> Bjerrum, N.; *Z. physik. Chem.*, 1923, civ, 147.

## EXPERIMENTAL.

*A. Preparation of Mixed Lysolecithin and Lysocephalin.*—A crude mixture of lysolecithin and lysocephalin was prepared by the general method described in the preceding paper of this series. Some slight modifications of this method have been found advantageous. Thus, the digestion of egg yolk was carried out in small lots, twenty yolks being diluted with 600 cc. of  $M/15$  phosphate solution of a  $pH = 7.0$ .<sup>5</sup> This volume was digested with 0.010 gm. of cobra venom for 14 hours at  $40^{\circ}C$ . The mixture was then poured into an equal volume of warm ( $40$ – $50^{\circ}C$ .) 95 per cent alcohol and the precipitated proteins were removed by filtration. As the protein material retained considerable quantities of hydrolyzed lipoids, it was exhaustively extracted with 95 per cent alcohol and the combined liquors were concentrated under diminished pressure to a small bulk. To the residue a concentrated aqueous solution of cadmium chloride was added. The mixed cadmium salts, precipitated by the addition of several volumes of acetone, were filtered after standing at  $0^{\circ}$  overnight. It is essential to remove the adhering oils at this point by thorough washing with acetone. The salts were then suspended in warm chloroform and decomposed with alcoholic ammonia. The precipitated cadmium hydroxide (A) is filtered off, the filtrate is concentrated, and to the residue enough ether is added to precipitate the mixed lysolecithin and lysocephalin. Purified by repeated solutions in chloroform and precipitation from acetone or ether, such material analyzed as follows:

0.1032 gm. substance: 0.2220 gm.  $CO_2$ , 0.0938 gm.  $H_2O$ , and 0.0147 gm. ash.

0.1880 " " : (Kjeldahl) 4.10 cc  $0.1\ N$  acid.

0.2820 " " : (fusion) 0.0522 gm  $Mg_2P_2O_7$ .

The amino distribution<sup>6</sup>

5 cc.: (Kjeldahl) 5.70 cc  $0.1\ N$  acid.

2 " : (Van Slyke) 1.29 "  $N_2$  at  $22^{\circ}C$ ., 750 mm.

Found No. 380. C 58.66, H 10.17, N 3.03, P 5.15, ash 14.24.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{22}{100}$$

<sup>5</sup> A series of similar experiments, carried out over a range of hydrogen ion concentrations varying from  $pH$  6.5 to 8, indicated that whereas cobra venom is active between  $pH$  6.5 and 7.5, it is entirely inactivated at  $pH$  8.

<sup>6</sup> Cf. Levene and Rolf,<sup>1</sup> p. 745.

Since abundant bacterial growths were found in the digestion mixture, the experimental conditions were varied in an attempt to attain sterility. All digestion mixtures, however, showed the presence of bacterial growth.

Table I shows the yields of the mixed lyso bodies when twenty eggs in 600 cc. of  $\frac{M}{15}$  phosphate solution (pH = 7.2) were digested at 40°C.

TABLE I.

Experiment No	Time of digestion.	Cobra venom	Antiseptic used.	Yield of lyso bodies
	hrs	mg		gm.
1	16	10	None.	3.7
18	16	10	"	6.3
2	16	10	2 drops toluene	6.4
19	16	10	2 " "	9.1
14	6	10	2 " "	1.9
15	6	5	2 " "	1.0
16	6	20	2 " "	9.0
20	2	20	2 " "	4.7
13	6	10	36 cc. "	2.5
10	6	10	6 gm. NaF.	0.15
9	3	10	6 " "	0.15
11	6	10	3 " "	2.0
12	6	5	6 " "	0.0
21	15	10	6 cc. $\text{CHCl}_3$ .	3.0
23	15	10	5 " " + 5 cc. toluene.	1.5
22	15	0	None	0.0

*B. Separation and Properties of Lysolecithin.*—If the chloroform solution, (A), after separation from the inorganic salt, was concentrated until a precipitate just appeared in the flask and then allowed to stand at room temperature, material (crystalline if adhering oils have been sufficiently removed from the cadmium salt) which contained 75 per cent of lysocephalin separated. From 500 gm. of crude cadmium salt 20 to 30 gm. of such material were obtained. By adding ether to the mother liquor until a faint turbidity developed and then allowing it to stand at 0°C., a second precipitate, containing 37 per cent of lysocephalin, was deposited.

Complete precipitation after the separation of this material yielded a lysolecithin fraction containing about 10 per cent of

lysocephalin. From 500 gm. of crude cadmium salt, the maximum yield of such a fraction was 100 gm. From this fraction material of a higher degree of purity was obtained by adding to a warm chloroform solution a quantity of cadmium chloride insufficient to precipitate completely the lysolecithin present in solution. Thus, 175 gm. of such a material containing 10 per cent of lysocephalin were dissolved in 1 liter of chloroform and 50 gm. of  $\text{CdCl}_2$  in alcoholic solution were added. 145 gm. of lysolecithin cadmium chloride containing 4 per cent of amino nitrogen were deposited. By the complete precipitation of the mother liquor with cadmium chloride, an additional precipitate of 5.0 gm. was formed. This precipitate contained 7 per cent of amino nitrogen. Repetition of this procedure yielded amino-free material.

From the fraction containing 37 per cent of amino nitrogen amino-free lysolecithin could be obtained, after several such partial precipitations.

No. 540

0.0944 gm substance · 0.2000 gm  $\text{CO}_2$ , 0.0887 gm  $\text{H}_2\text{O}$ , and 0.0130 gm ash.

0.1802 " " : (Kjeldahl) 3.70 cc. 0.1 N acid.

0.2712 " " : (fusion) 0.0578 gm.  $\text{Mg}_2\text{P}_2\text{O}_7$ .

It contained no amino nitrogen

Found. No. 540. C 57.77, H 10.45, N 2.89, P 5.96, ash 13.77.

Calculated. Lysolecithin.  $\frac{\text{Amino N}_2}{\text{Total N}_2} = \frac{0}{100}$

Containing stearic acid. C 57.65, H 10.43, N 2.59, P 5.73.

" palmitic acid. " 56.10, " 10.29, " 2.72, " 6.14.

This material was readily soluble in hot pyridine from which, on cooling, it crystallized practically quantitatively in aggregates of fine needles. When thoroughly dry, it formed a pure white powder. However, unlike lysocephalin, it is very hygroscopic. It analyzed as follows:

No. 520.

0.1020 gm. substance: 0.2136 gm.  $\text{CO}_2$ , 0.0920 gm.  $\text{H}_2\text{O}$ , and 0.0150 gm. ash.

0.1932 " " : (Kjeldahl) 4.00 cc. 0.1 N acid.

0.2898 " " : (fusion) 0.0632 gm.  $\text{Mg}_2\text{P}_2\text{O}_7$ .

It contained no amino nitrogen.

Found. No. 520. C 57.10, H 10.09, N 2.89, P 6.08, ash 14.70.

Crystalline lysolecithin is readily soluble in, and may be recrystallized from, chloroform, glacial acetic acid, pyridine, and

methyl and ethyl alcohols. It is insoluble in ether, gasoline, and acetone. With water it forms an emulsion typical of lipoidal materials. The addition of alkali causes immediate solution. When this solution is reacidified and allowed to stand, it sets to a colorless gel.

When heated, it softens slightly at about 100°C. Further heating does not materially change its appearance before it melts with gas evolution and blackening at 263°C.

Its rotation in chloroform solution was

$$[\alpha]_D^{20} = \frac{-0.26^\circ \times 100}{1 \times 10} = -2.6^\circ$$

In pyridine solution it was

$$[\alpha]_D^{25} = \frac{+0.12^\circ \times 100}{1 \times 10} = +1.2^\circ$$

In glacial acetic acid it was

$$[\alpha]_D^{25} = \frac{+0.08^\circ \times 100}{1 \times 10} = +0.8^\circ$$

*C. Separation and Properties of Lysocephalin.*—The yield of crude lysocephalin, separated by its insolubility in chloroform from the first mixture of lysolecithin and lysocephalin, could be augmented by a fractionation of the intermediate fractions. Thus, the mother liquors, from which lysolecithin had been separated as the cadmium chloride salts, were combined and concentrated under diminished pressure. To the residue an aqueous solution of cadmium chloride was added and precipitation completed by the addition of several volumes of acetone. This mixed salt had a ratio of amino nitrogen to total nitrogen of 35:100. It was dissolved in chloroform and decomposed with alcoholic ammonia according to the usual technique. On concentrating the mother liquor *in vacuo*, a precipitate, rich in lysocephalin, was gradually deposited in the flask. Recrystallized from chloroform or pyridine, this material analyzed as follows:

No. 395.

0.1068 gm. substance: 0.2208 gm. CO<sub>2</sub>, 0.0950 gm. H<sub>2</sub>O, and 0.0166 gm. ash.

0.1996 " " : (Kjeldahl) 4.45 cc. 0.1 N acid.



0.2995 gm. substance : (fusion) 0.0704 gm.  $Mg_2P_2O_7$ .

0.2 gm. dissolved in glacial acetic acid.

5 cc. : (Kjeldahl) 1.50 cc. 0.1 N acid.

2 " : (Van Slyke) 1.51 "  $N_2$  at 22°C., 755.2 mm.

Calculated. Lysocephalin.  $\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{100}{100}$

Containing stearic acid. C 57.35, H 10.05, N 2.91, P 6.44.

" palmitic acid. " 55.58, " 9.80, " 3.06, " 6.84.

Found. No. 395. C 56.37, H 9.94, N 3.12, P 6.55, ash 15.54.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{100}{100}$$

Three other samples of crystalline lysocephalin obtained from different preparations analyzed as follows:

No. 350 C 56.55, H 10.27, N 3.10, P 6.49, ash 15.40.

" 395 B. " 56.33, " 9.9, " 3.08, " 6.50, " 15.55.

" 539. " 56.38, " 10.00, " 2.99, " 6.49, " 14.45.

Lysocephalin is a gleaming white powder, showing no tendency to absorb moisture on exposure to the air. From chloroform, it crystallizes in flat, rather transparent needles. It is difficultly soluble in glacial acetic acid and in hot pyridine. When pure, it is almost insoluble in chloroform and insoluble in ether and acetone. It may be dissolved in hot absolute methyl alcohol, from which it precipitates crystalline on the addition of water. With water it forms an emulsion, which dissolves immediately on the addition of sodium hydroxide. Acidification, however, causes the instantaneous formation of a very stiff colorless gel.

When heated, for a melting point, No. 395 softened slightly above 140°C., melting at 198°C. After cooling and remelting, it again softened and melted sharply at 203–204°C. Recrystallized from pyridine it melted sharply at 212–213°C.

Owing to its insolubility its rotations in pyridine solution and in chloroform solution could not be taken.

In glacial acetic acid its rotation was

$$[\alpha]_D^{25} = \frac{+0.20^\circ \times 100}{1 \times 10} = +2.0^\circ$$

*Fatty Acids of Lysolecithin.*

20 gm. of lysolecithin were heated in an autoclave at 105° with 3 per cent  $\text{H}_2\text{SO}_4$ . The hydrolysis of this substance was effected with greater difficulty than in the case of lysocephalin, and was complete only after 8 hours. The fatty acids were filtered, dissolved in ether, and thoroughly washed. A yield of 10.0 gm. of crude material was obtained, which melted at 57°C. From these acids the methyl esters were prepared and the latter were fractionated by distillation at a pressure of 0.7 mm.

The esters were saponified, and analyses, melting points, and molecular weight determinations were made on the free acids in the manner described in previous publications.<sup>7</sup>

No. 561.

0.1000 gm. substance: 0.2756 gm.  $\text{CO}_2$  and 0.1114 gm.  $\text{H}_2\text{O}$ .

0.7913 " " required for neutralization 6.1 cc. 0.5 N NaOH.

No. 562.

0.1006 gm. substance: 0.2772 gm.  $\text{CO}_2$  and 0.1130 gm.  $\text{H}_2\text{O}$ .

0.9032 " " required for neutralization 6.6 cc. 0.5 N NaOH.

No. 563.

0.997 gm. substance: 0.2770 gm.  $\text{CO}_2$  and 0.1156 gm.  $\text{H}_2\text{O}$ .

0.8355 " " required for neutralization 5.9 cc. 0.5 N NaOH.

No. 564.

0.1002 gm. substance. 0.2794 gm.  $\text{CO}_2$  and 0.1152 gm.  $\text{H}_2\text{O}$ .

0.7182 " " required for neutralization 5.05 cc. 0.5 N NaOH.

TABLE II.

Nos 561 and 562 were the redistilled fractions of the lowest boiling fraction.

	No.	Boiling point of ester at 0.7 mm	Analysis of acid		Molecular weight of acid	Melting point of acid
			C	H		
Found.	561	140-145	75.15	12.47	259	62
"	562	146-150	75.14	12.56	273	58-59
"	563	153	75.76	12.97	282	65-66
"	564	156-160	76.03	12.88	284	70-71
Calculated. $\text{C}_{18}\text{H}_{32}\text{O}_2$ . . . .			74.92	12.58	256	63
$\text{C}_{18}\text{H}_{34}\text{O}_2$ . . . . .			75.98	12.76	284	71

<sup>7</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlv, 363; 1922, li, 507.

*Fatty Acids of Lysocephalin.*

20 gm. of lysocephalin were hydrolyzed, esterified, and fractionated in the same manner as was lysolecithin. Hydrolysis was complete in 4 hours. 10 gm. of the crude fatty acid were obtained, melting at 61–62°C.

Three fractions were obtained, of the characteristics described below.

No. 551.

0.1003 gm. substance: 0.2792 gm. CO<sub>2</sub> and 0.1162 gm. H<sub>2</sub>O.

0.8221 " " required for neutralization 5.85 cc. 0.5 N NaOH.

No. 552.

0.1003 gm. substance: 0.2798 gm. CO<sub>2</sub> and 0.1156 gm. H<sub>2</sub>O.

0.8658 " " required for neutralization 6.10 cc. 0.5 N NaOH.

No. 553.

0.1001 gm substance: 0.2781 gm. CO<sub>2</sub> and 0.1130 gm. H<sub>2</sub>O.

0.8739 " " required for neutralization 6.15 cc. 0.5 N NaOH.

TABLE III

	No	Boiling point of ester at 0.7 mm	Analysis of acid.		Molecular weight of acid	Melting point of acid
			C	H		
Found.	551	150–152	75.90	12.96	280	65
"	552	152–155	76.03	12.98	283	69.2
"	553	155–158	75.76	12.63	284	70–71
Calculated	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>		74.92	12.58	256	63
	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>		75.98	12.76	284	71

*Dissociation Constants of Lysolecithin and Lysocephalin.*—Lysolecithin and lysocephalin were titrated potentiometrically and their dissociation constants calculated in accordance with the formulas given below. Each sample was dissolved in the requisite amount of acid or alkali and diluted to the concentration designated. The pH was determined with the apparatus previously described.<sup>8</sup>

When titrating with a strong base the formula

$$\text{pK}_2 = \text{pH} + \log \frac{1 - B'}{B'}$$

is used.

<sup>8</sup> Simms, H. S., *J. Am. Chem. Soc.*, 1923, xlv, 2503.

When titrating with a strong acid the value is determined by the formula

$$pK_1 = pH + \log \frac{A'}{1 - A'}$$

where,

$$B' = \frac{B}{C} - \frac{OH}{C} \quad \begin{array}{l} \text{Equivalents of acid or base added, corrected} \\ \text{for acid or base required to bring water to} \\ \text{the same pH (assuming complete ionization} \\ \text{of the strong acid or base).} \end{array}$$

$$A' = \frac{A}{C} - \frac{H}{C}$$

B = Molar concentration of base added.

A = " " " acid "

C = " " " substance.

H = " " " H<sup>+</sup> ions.

OH = " " " OH<sup>-</sup> ions.

TABLE IV.  
*Lysolecithin, pK<sub>1</sub>.*

C	$\frac{A}{C}$	pH	$\frac{H}{C}$	A'	$\log \frac{A'}{1 - A'}$	pK <sub>1</sub>	K <sub>1</sub>
0 050	0 500	1 70	0 400	0 100	-0 95	0 75	0 18
(0 05)	(0 90)	1 45	0 71	0 19	-0 63	(0 82)	(0 15)
0 050	1 000	1 39	0 814	0 186	-0 65	0 74	0 18
Probable value. . . . .						0 75	0.18

TABLE V.  
*Lysolecithin, pK<sub>2</sub> (pK<sub>w</sub> is taken as 13 77)*

C	$\frac{B}{C}$	pH	pOH	$\frac{OH}{C}$	B	$\log \frac{1 - B'}{B'}$	pK <sub>2</sub>	K <sub>2</sub> × 10 <sup>13</sup>
0 100	0 500	11.74	2 03	0 093	0 407	+0 16	11 90	1 26
0 100	1 200	12 32	1 45	0 355	0 845	-0 73	(11 6)	(2 5)
0 050	0 600	11 37	2 40	0 079	0.521	+0 04	(11 4)	(4)
0 050	0.800	12 00	1 77	0 340	0 460	-0 07	11 93	1.18
0 050	0 200	11 13	2 64	0 046	0 154	+0 74	11 87	1 35
Average of best values . . . . .							11.90	1 26

### *Lysocephalin, pK*

Due to the immediate formation of a gel in acid solution,<sup>9</sup> the value of the lower pK could not be accurately determined.

<sup>9</sup> The rapidity with which lysocephalin forms a gel is quite remarkable. When a cold solution of this substance is quickly mixed with dilute acid a solid gel is formed in a fraction of a second.

An approximate value was obtained from the pH value of such a gel containing 0.9 equivalent of acid. This value was obtained colorimetrically by the drop method and is only approximate.

$$\text{pH} = 2.5, \log \frac{A'}{1-A'} = +1.0, \text{pK}_1 = 2.5 + 1.0 = 3.5$$

$$\text{K}_1 = 3 \times 10^{-4} \text{ (approximate).}$$

TABLE VI.  
*Lysocephalin*,  $\text{pK}_2$  ( $C = 0.05$  molar.)

B'	pH	$\log \frac{1-B'}{B'}$	$\text{pK}_2$	$\text{K}_2 \times 10^{10}$	Remarks
0.90	10.6	1.0	9.6	2.5	pH value not definite.
0.95	10.6	1.3	9.3	5	" " " "
0.95	10.5	1.0	9.5	3	" determined on hot solution.
Average			9.47	3.4	

TABLE VII  
*Summary of Results*

	$\text{pK}_1$	$\text{pK}_2$	$\text{K}_1 \times 10^2$	$\text{K}_2 \times 10^{11}$	Isoelectric range	Isoelectric point.
Lysolecithin	0.75	11.90	18	0.13	2.75-9.90	6.33
Lysocephalin	(3.5)	9.47	0.03	34	5.5-7.5	6.5

Each of these compounds has two pK values. One pK corresponds to the dissociation constant of the free acid group of the phosphoric acid radical. The other corresponds to  $\frac{K_w}{K_b}$  where  $K_w$  equals the dissociation constant of water and  $K_b$  equals that of the basic group (in the amino ethanol or choline).

*Hemolytic Action of Lysolecithin and Lysocephalin.*—Through the kindness of Dr. Hideyo Nogouchi, the hemolytic activity of samples of lysolecithin and lysocephalin toward horse cells was tested. While in both substances this quality was of the same order of magnitude, lysolecithin showed an activity three times as great as lysocephalin, as shown by the protocol given below.

The solutions used were  
No. 1.

0.100 gm. lysolecithin dissolved in 10 cc. methyl alcohol.

No. 2.

0.100 gm. lysolecithin suspended in 10 cc. H<sub>2</sub>O containing one equivalent of NaOH.

No. 3.

0.100 gm. lysocephalin suspended in 10 cc. methyl alcohol. It remained practically undissolved.

No. 4.

0.100 gm lysocephalin dissolved in 10 cc. H<sub>2</sub>O containing one equivalent of NaOH.

TABLE VIII.

Dilution.	Lysolecithin.		Lysocephalin.	
	No 1	No 2	No 3	No. 4
Undiluted.	C. H.	C. H.	Sl. H.	C. H
1:10				
0 1 cc.	C. H.	C. H.	No H.	C. H.
1:100				
0 7 cc.	C. H.	C. H.		C. H.
0 5 "	"	"		"
0 3 "	"	"		Almost C. H.
0 2 "	"	"		Sl. H.
0 1 "	"	"		No H.
1:1,000				
0 7 cc.	No H.	No H.		

C H. = complete hemolysis, Sl H. = slight hemolysis; No H. = no hemolysis.



# A METHOD OF DETERMINING THE BIOLOGICAL VALUE OF PROTEIN.

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Probably the most direct method of measuring the degree to which the animal body utilizes dietary protein for those purposes for which this class of nutrients alone can serve is by means of a study of the nitrogenous metabolism under certain definite conditions. These conditions are: first, that in so far as possible the diet contains only that protein or mixture of proteins which it is desired to investigate; second, that in so far as possible the diet contains no non-protein nitrogenous substances except those present in the food under investigation; and, third, that the food intake be so adjusted as to composition and amount that dietary protein will not be used as a source of energy except in so far as such disposition is conditioned by incomplete utilization for the maintenance of the nitrogenous integrity of the tissues, for their growth, or for the elaboration of milk. It would seem to be legitimate to investigate the value of a food in covering the body's requirements for nitrogen, even though a considerable fraction of the nitrogen content of the food is in non-protein forms. The biological value obtained in such a case should, of course, refer to the total nitrogen of the food, not to its total protein.

However, even when these conditions are satisfactorily met, the interpretation of the data of the nitrogenous metabolism relative to the purpose for which they have been obtained is not simple, being complicated by the fact that both urinary and fecal excretions contain nitrogen resulting from tissue catabolism or unavoidable tissue wastage, as well as the nitrogen resulting from the incomplete digestibility or the subsequent incomplete utilization of the dietary protein. Since it is the purpose of the experiment to measure the wastage of dietary nitrogen in diges-



tion and metabolism, the body's contribution of nitrogen to the urinary and fecal excretions must be assessed. Unfortunately, no direct method is available, so that indirect methods, admittedly of uncertain accuracy, have been used. It will be the purpose of this paper to consider critically the validity of these indirect methods, utilizing for this purpose metabolism data obtained on rats in the Nutrition Laboratory of the University of Illinois.

Since the conviction is often expressed that accurate results on the nitrogenous metabolism of small animals, such as rats, cannot be obtained, it seems advisable to describe the method used in this work and to give the results of the many rigorous tests of its accuracy performed in this laboratory. As a result of these tests, the conclusion seems warranted that the method possesses a high degree of accuracy.

*A Method for Nitrogenous Metabolism Investigations on Rats.*

The rats were placed in large crystallizing dishes measuring 190 mm. in diameter and 100 mm. in depth. Weighted wire covers were placed on top. The food crucibles were held in wire frames suspended from the covers. Distilled nitrogen-free water was provided by an inverted bottle or test-tube supported on the wire cover with its bent glass tube projecting into the dish. On the floor of the dish two or three layers of filter paper, cut to fit the dish closely, were placed to absorb the urine. A unit of ten of these metabolism dishes may be kept in a two-shelf box with a wire screen back and screen doors in front to permit efficient ventilation and to exclude the possibility of error from the presence of flies or wild mice. Proper ventilation of these dishes may be insured by the use of an electric fan.

All the rations used were mixed thoroughly in the wet, generally steam-cooked, except for the vitamin preparations which were added later, spread out in thin layers on glass plates, and dried on steam radiators at low temperatures or before a blast of warm air. They were then broken up, ground fine in the laboratory mill, dried for several days in an oven at 40–50°C., and analyzed for total nitrogen.

In practically all the experiments to be reported the metabolism periods were of 1 week duration, during which time the urine and feces were collected daily. In changing from one ration to

another at least 3 days elapsed before urine and feces were again collected and analyzed. Body weights were taken at the beginning and end of each balance period.

The food was weighed out each day into the crucibles and mixed with a little water to a thick consistency to prevent scattering. The next day any residue left was scraped out and dried in the same oven and at the same temperature as the original ration. The food residues for each week were weighed after several days drying, and the weight was subtracted from the total food weighed out to determine the food consumed per week. By several tests, it was shown that the error introduced into this determination, due to a possible difference in moisture content of the original ration and of the residues, was less than 1 per cent. It was also determined that the percentage of nitrogen in these food residues was identical with that of the ration from which they were taken.

Urine and feces were collected daily. The feces were preserved under 95 per cent alcohol, containing 10 per cent thymol, or in the later experiments, a few drops of sulfuric acid or hydrochloric acid. The dish and paper of each rat were washed thoroughly with boiling water, acidified strongly with sulfuric (or hydrochloric) acid. About 250 cc. of wash water in four to six portions were used on each dish. The washings were filtered through glass-wool into 250 cc. volumetric flasks, which were then cooled in the ice box overnight. The next day they were made up to the mark at ice box temperature and emptied into 2.5 liter bottles in which the weekly composite samples were kept. The bottles contained crystals of thymol and were kept in a cold storage room until analyzed. Samples of 50 to 250 cc., measured out in the cold, were analyzed for total nitrogen.

The week's collection of feces was digested with sulfuric acid, mercury, and potassium sulfate until completely oxidized. The residue was then transferred to a volumetric flask, and aliquots were taken for distillation.

The accuracy of the method used in determining the excretion of nitrogen in the urine was the subject of considerable study, in part because the determination of the biological value of protein is so largely dependent upon an accurate estimation of the urinary nitrogen, and in part because the results of metabolism experi-

ments on small animals are so frequently discounted by the belief that accurate balance data on such subjects cannot be expected, possibly because of the small amounts of nitrogen involved.

The filter paper used to absorb the urine was found to contain only small amounts of nitrogen, one piece of the size used in the metabolism experiments containing about 0.4 mg. of nitrogen. Since two pieces were used generally in each dish per day, the error thus contributed amounted to 0.8 mg. at most. In so far as this was insoluble in dilute acid, it would not affect the results one way or the other. In so far as it was soluble, it would dimin-

TABLE I  
*Completeness of Extraction of Urinary N from the Filter Papers Used as Absorbents.\**

Total urinary nitrogen determined	Extractable nitrogen remaining in paper residues	Error
<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
918	26	2 8
913	25	2 7
826	16	2 0
860	15	1 7
627	10	1 6
619	8	1 3
551	14	2 5
576	14	2.4
567	8	1 4
398	8	2 0
181	2 5	1.4
174	2 5	1.4

\* Results expressed in mg. per week.

ish the error due to the incomplete extraction of the urinary nitrogen from the filter paper.

The completeness of extraction of urine from the filter paper by the 250 cc. of hot dilute acid used was determined by saving the paper residues from several balance periods, extracting each with 2 or 3 liters of boiling water, acidified with sulfuric acid, filtering, evaporating the extract to a small volume, transferring to a Kjeldahl flask, and determining nitrogen in it. The results thus obtained compared with the quantities of urinary nitrogen determined in the corresponding balance periods are given in Table I.

The error due to the retention of urine by the filter paper residues is evidently negligible in biological work.

A more thorough test of the method of collecting urines was made by adding known amounts of urea to a ration, the contributions of which to the urinary and fecal nitrogen had been determined in two preceding periods. The completeness of recovery of the urea was then determined. The subjects of this test were two rats which had been on a yeast ration for 2 weeks. Following two balance periods on this ration, definite volumes of a standard solution of urea (Kahlbaum) were added to each day's portion of food. As in the preceding tests, the residues of each day's food were collected, dried, and weighed. In this test they

TABLE II  
*Recovery of Urea Fed in Urine Collected.\**

Rat No	Average body weight	Period	Food intake	Food nitrogen	Urea nitrogen added	Urinary nitrogen	Fecal nitrogen
	<i>gm.</i>		<i>gm</i>	<i>mg</i>	<i>mg</i>	<i>mg.</i>	<i>mg.</i>
24	97	10	41 7	365	0	217	97
	101	11	47 7	417	0	226	113
	105	12	49 1	430	110	353	143
25	61	10	36 1	316	0	202	64
	62	11	45 7	400	0	216	125
	64	12	35 3	309	85	289	105

\* Results expressed on weekly basis.

were analyzed for nitrogen also. 2 days after the addition of urea to the food, the collection of urine and feces began and continued for a week, with the results shown in Table II.

Comparing Periods 11 and 12 for Rat 24, during which comparable amounts of food were consumed, the addition of 110 mg. of urea nitrogen per week to the yeast ration caused an increase of 127 mg. in the excretion of urinary nitrogen as determined by the method under examination. Similarly, with Rat 25, comparing Periods 10 and 12, an addition of 85 mg. of urea nitrogen to the week's rations raised the urinary excretion of nitrogen 87 mg. In both cases, the addition of urea to the ration seemed to depress the digestibility of the yeast protein. The results, however, seem to indicate that the added urea was quanti-

tatively recovered from the products of the filter paper used to absorb the urine.

For further accuracy of the determinations of urinary and fecal nitrogen, reference is made to a paper by Nevens, published in the *Laboratory* (1). These tests indicate errors of only 1% in the extraction of urine nitrogen from the filter paper. They also indicate clearly that no losses of nitrogen occur in 4 to 48 hours due to bacterial decomposition of feces exposed in open dishes even at a temperature

*Determination of the Amount of Fecal Nitrogen Derived from the Body, the So Called Metabolic Nitrogen of the Feces.*

The "biological value" of a protein, as the term was applied originally by Karl Thomas, referred to the utilization by the body of the products of protein digestion. The biological value was expressed as the percentage of the absorbed nitrogen which was retained by the body for repair or the construction of nitrogenous tissue. Hence, differences in digestibility of different proteins did not affect their biological values.

The determination of the amount of protein digested and absorbed from a given protein intake is complicated by the difficulty of distinguishing between fecal material derived from the food and that derived from the intestinal tract and its secretions. Obviously, only the former should be deducted from the protein intake to obtain the amount of protein absorbed. While various methods have been devised for making this separation of fecal nitrogen by digestion of the feces with enzymes or special solvents, the assumptions upon which such methods have been based are not particularly convincing and the technique involves conditions of time and temperature quite arbitrarily imposed. Thomas preferred to measure the so called "metabolic nitrogen" of the feces by the daily fecal excretion of nitrogen from a diet practically free of this element, and more recently Martin and Robison (2) have accepted this procedure in their own calculations. The assumption has been, however, that the excretion of metabolic nitrogen is constant regardless of the amount of food consumed, though the latter investigators were fully aware of the fact that the composition of the diet, in particular its content of indiges-

tible material, may greatly affect the excretion of metabolic products in the feces. They believe that the determination of the metabolic nitrogen of the feces is the factor limiting the accuracy of biological values of protein by the method of Thomas.

That the amount of roughage in the diet is a factor determining the amount of metabolic nitrogen appearing in the fecal excretion is demonstrated by some results obtained with four rats of the same litter subsisting on a ration containing 15 per cent of butter fat, 14 per cent of lard, 66 per cent of starch, 4 per cent of inorganic salts, and 1 per cent of an alcohol extract of carrots as a source of vitamin B. Starting on the 4th day of feeding, the

TABLE III.

*Effect of Roughage on the Amount of Metabolic Nitrogen in the Feces.*

Rat No	Initial weight	Final weight	Food eaten per day	Urinary nitrogen per day	Fecal nitrogen per day	Fecal nitrogen per 100 gm. food.	
						With filter paper.	Without filter paper.
	gm	gm	gm	mg	mg	mg	mg.
1	91	86	4 84		6 0		124
	86	79	4 43	11 7	7 9	180	
2	88	82	4 26		4 7		111
	82	79	4 21	9 8	6 9	164	
3	99	95	4 94		6 4		130
	95	90	5 16	11 2	8 2	159	
4	97	90	4.44		5 5		123
	90	86	4 21	12 3	8 0	191	
Average.....						173	122

feces were collected for the succeeding week during which time the rats had no access to filter paper. Following this period was one in which the same ration was used, but in which the rats had free access to filter paper and actually consumed some of it. In the latter period the urine was also collected and analyzed for nitrogen. The essential figures are given in Table III.

During the periods in which filter paper was consumed, the fecal nitrogen in all cases increased, the increase in the average amounting to 42 per cent. Therefore, if the fecal excretion on a nitrogen-free diet is to be taken as the measure of the excretion of metabolic nitrogen in the feces of subsequent feeding periods

with diets containing protein, it would be advisable to equalize the crude fiber content of the non-nitrogenous and nitrogenous diets. However, in the metabolism experiments with rats, a variable consumption of the filter paper used for the collection of urine occurred in all periods, so that equalizing the crude fiber content of the experimental rations would not serve its purpose. An unavoidable error enters into the method here, but is not

TABLE IV.

*Influence of the Amount of Food Consumed on the Excretion of Fecal Nitrogen with Rations Practically Nitrogen-Free.*

Rat No.	Average weight	Daily food intake	Daily excretion of urinary nitrogen	Daily excretion of fecal nitrogen	
				Total.	Per gm. of food
	gm	gm	mg	mg	mg.
1	218	11 32	24 5	24 3	2 15
	213	7 38	28 6	18 3	2 48
2	266	14 24	26 6	35 5	2 49
	257	8 67	37 7	21.7	2 51
3	162	10 41	13 2	18.9	1 81
	156	6 00	22 2	11.3	1 88
4	145	9 61	12 9	21 0	2.19
	139	6 00	17 4	11 3	1.88
5	149	8 73	21 0	15 5	1 78
	143	5 93	22 0	10 7	1.78
6	69	5 00	8.3	9 5	1 90
	65	3 60	9 3	5 5	1 53

thought to be serious, since an underestimation of the metabolic nitrogen in a period of protein feeding would lead to an overestimation both of the food nitrogen retained in the body and of the absorbed nitrogen, the numerator and the denominator, respectively, of the fraction determining the biological value sought after.

It may be readily demonstrated that the excretion of nitrogen in the feces on diets containing minimal amounts of nitrogen

(0.04 to 0.08 per cent) varies directly with the amount of food consumed. The experimental results in Table IV bear on this point.

Six rats of different weights were fed *ad libitum* on a nitrogen-free ration. After a preliminary period of 3 days, the urine and feces were collected quantitatively for 7 days. At the end of this time the food intake was restricted to an amount corresponding to their energy requirement for maintenance as previously determined. A 7 day collection period at this lower level was then run, with no transition period. The average daily fecal nitrogen dropped in all cases in the second period, and, as the last column of figures shows, the decrease was closely proportional to the decrease in food consumed for three of the rats; while the relation was not so close for the other three rats, the divergence from a linear relation might well be due to the variation to be expected in biological experiments.

From these and many similar results we have concluded that the best and most practicable method of estimating the metabolic nitrogen in the feces of rats on an experimental ration containing protein is to determine in a period of feeding a non-nitrogenous ration, the excretion of fecal nitrogen per gram of food consumed, and to apply this figure to the amount of protein-containing food consumed in subsequent experimental periods. While many objections may be raised to this procedure, it seems to be the best method available, and should be judged, not by the errors theoretically possible, but by the consistency and plausibility of the results obtained.

Six rats of widely differing weights were fed for several months on amounts of food just sufficient to maintain constant weight. The protein in the rations fed during this time was derived entirely (except for unavoidable impurities in the starch, sugar, butter fat, and small concentrations of the vitamin B preparation of Osborne and Wakeman) from a mixture of corn and tankage in the proportion of 85 parts of the former to 15 parts of the latter. The level of protein intake was varied by diluting this mixture of corn and tankage with varying amounts of a ration complete in every respect except for the entire absence of protein. From the different periods of this experiment, which was carried out with another object in view, it is possible to pick out periods in



which all the rats were consuming practically the same amount of this constant source of protein. These periods varied in length from 7 to 20 days. The data pertaining to the digestibility of the rations during these periods are given in Table V.

The estimates of the metabolic nitrogen in the feces given in Column 6 were obtained from the fecal nitrogen excreted on like amounts of a protein-free ration (see Table IV, the second period).

Due to the inconstant proportion of metabolic nitrogen in the fecal nitrogen excreted by these rats, the coefficients of apparent digestibility vary from 51.7 for the heaviest rat to 77.0

TABLE V.

*Digestibility of the Proteins of a Ration of Corn and Tankage by Rats of Different Weights. Result of Correcting the Digestibility for the Metabolic Nitrogen of the Feces*

Rat No	Average weight	Daily food consumption	Daily intake of nitrogen	Daily fecal nitrogen	Estimated metabolic nitrogen in feces	Percentage digestibility of protein.	
						Uncorrected	Corrected.
	<i>gm</i>	<i>gm</i>	<i>mg</i>	<i>mg.</i>	<i>mg.</i>		
2	262	8 70	56 9	27 6	21 7	51 7	89 6
1	213	7 50	60 0	23 1	18 3	61 5	92 0
3	155	6 00	62 6	18 1	11 3	71 0	89.1
4	145	6 00	62 6	19 0	11.3	69 6	87.7
5	145	6 00	62 6	20 9	10.7	66.6	83.7
6	57	3 60	55 1	12 7	5 5	77 0	86.9

for the lightest. When allowance is made, however, for the body nitrogen excreted in the feces, the digestibility thus corrected ranges only from 83.7 to 92.0 per cent, with no obvious relation to body weight.

Practically the same situation is shown with three other rats placed successively on rations containing approximately 5 and 10 per cent of corn protein. The metabolic nitrogen in the feces was measured in a following period on a nitrogen-free ration. The data on digestibility are summarized in Table VI.

The uncorrected coefficients are evidently seriously vitiated by varying proportions of body nitrogen in the feces. When the amounts of nitrogen of intestinal origin are estimated by the total fecal nitrogen excretion on nitrogen-free rations, and these

TABLE VI.

*Digestibility of the Proteins of Corn at Different Levels of Intake, Corrected and Uncorrected for the Metabolic Nitrogen of the Feces.*

Rat No.	Protein.	Concentration (approximate).	Digestibility of protein.	
			Uncorrected.	Corrected.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
60	Corn.	5	66.6	90.8
	"	10	79.7	92.1
	"	10	77.8	90.2
61	"	5	66.7	89.8
	"	5	64.4	87.5
	"	10	80.9	92.7
	"	10	80.5	92.3
62	"	5	67.7	87.9
	"	10	80.6	90.9
	"	10	76.8	87.2

TABLE VII.

*Digestibility of Protein for Rats.*

In concentrations of approximately 5 per cent

In concentrations of approximately 10 per cent.

No of rats	No. of periods.	Kind of protein	Digestibility		No of rats	No of periods	Kind of protein.	Digestibility.	
			Uncorrected	Corrected				Uncorrected.	Corrected.
6	9	Beef protein.	75	94	2	5	Beef protein.	88	99
5	10	Milk "	76	97	5	10	Milk "	83	95
7	22	Casein.	74	93	3	26	Casein.*	83	90
3	4	Lactalbumin.	76	92					
4	13	Yeast protein.	59	78	5	20	Yeast protein.	63	76
4	12	Soy bean "	72	91	4	28	Soy bean " *	77	84
11	22	Corn "	73	95	12	20	Corn "	81	94
6	12	Oats "	70	90					
6	12	Rice "	77	96					
5	9	Potato "	59	78	5	15	Potato protein.	79	89
6	11	Navy bean "	61	76	5	13	Wheat "	79	94

\* These rations contained approximately 15 per cent of protein.

estimations used in the calculation of true digestibility coefficients, closely comparable values are obtained for all periods.

A summary of similar results for a number of different protein mixtures is given in Table VII.

An apparently significant difference in digestibility of the 5 and 10 per cent rations existed with soy bean and potato proteins. With the other proteins studied the differences in apparent digestibility indicated by the ordinary coefficients were practically elim-

TABLE VIII  
*Fecal Nitrogen per Gram of Food Consumed in Successive Periods of Non-Protein Feeding.*

Rat No	Length of period	Average weight of rat.	Average daily food intake	Average daily fecal nitrogen	Fecal nitrogen per day per gm. of food.
	<i>days</i>	<i>gm</i>	<i>gm</i>	<i>mg.</i>	<i>mg.</i>
9	4	103	4 82	8 4	1 75
	7	97	5 74	9 8	1 71
11	4	86	4 17	6 6	1 59
	4	82	3 90	7 6	1 95
12	7	104	4 85	8 9	1 84
	7	97	5 76	9 3	1 62
15	8	100	4 44	8 2	1 85
	7	91	4 16	8 6	2.08
16	7	104	5 62	9 4	1 68
	7	97	4 37	9 0	2 07
27	7	168	10 89	15 2	1.40
	7	164	7 81	14 6	1 87

inated when allowance was made for the metabolic nitrogen of the feces as measured by the fecal nitrogen excreted on a nitrogen-free ration

Rats will ordinarily excrete from 1.5 to 3.0 mg. of nitrogen per gram of nitrogen-free ration consumed. There is, however, a great deal of variation among rats in this respect. There seems to be a slight positive correlation between this value and the weight of the rat, while if the amounts of food consumed are

small compared to the energy requirement of the rat, abnormally large values are obtained which are obviously poor indices of the excretion of metabolic nitrogen in the feces under normal conditions of feeding.

The data in Table IV and those in Table VIII illustrate the agreement to be expected in successive periods of non-protein feeding.

TABLE IX

*Fecal Nitrogen per Gram of Food Consumed in Periods of Non-Protein Feeding Several Weeks Apart.*

Rat No.]	Experimental period	Average weight of rat.	Average daily food intake	Fecal nitrogen per day per gm. of food.
		gm.	gm	mg.
65	1	58	5 77	1.42
	10	131	5 80	1.82
	15	153	7 26	2 50
66	1	45	4 70	1 52
	10	105	4 36	2 20
	15	116	5 11	2 11
67	1	54	4 81	1.38
	10	120	5 17	2.25
	15	143	6 14	2.21
68	1	58	5 96	1 41
	10	115	4 51	2 63
	15	134	6 90	2 62
69	1	59	5 43	1 58
	10	111	5 24	2 30
	15	121	7 63	1.79

During an extended series of balance periods on the same rat, it is advisable to redetermine the metabolic nitrogen in the feces about every 6 weeks at most. At times unaccountable variations in this value will be revealed by these repeated tests, as shown by the data summarized in Table IX. All collection periods were of 7 days duration.

Evidently between the first and the tenth periods of this experiment a marked change in the intestinal conditions of these rats occurred, leading to an increased excretion of body nitrogen in

the feces. In using such results for the estimation of metabolic fecal nitrogen in the intervening eight periods it is necessary to assume a linear increase with time from the low level of excretion of the first period to the high level of the tenth period. From the tenth to the fifteenth experimental period, this high level was maintained well for three of the five rats.

*Determination of the Amount of Urinary Nitrogen of Body Origin.*

Having obtained a value for the absorbed nitrogen from a given nitrogen intake, the next step in measuring the biological value of dietary protein is to determine how much of the absorbed nitrogen is retained in the body. For most purposes it is sufficient to determine how much of the absorbed nitrogen is excreted in the urine, since the excretion of nitrogen by other paths is insignificant. The determination of the amount of nitrogen resulting from the catabolism of end-products of digestion of dietary protein is complicated by the fact that the urine is known to contain nitrogenous substances resulting from the catabolism of body tissues, as well as nitrogenous end-products of the catabolism of dietary protein. Since the nitrogenous end-products of these two types of catabolism, the "endogenous" and "exogenous" catabolism of Folin, are not to be clearly distinguished chemically they must obviously be determined by an indirect method.

The only conceivable indirect method of measuring the urinary nitrogen of endogenous origin is to eliminate entirely the exogenous catabolism by the feeding of a nitrogen-free diet, and insuring that enough of such a diet is consumed to provide sufficient energy for the requirements of the experimental animal, thus preventing the oxidation of body tissue for this purpose with its inevitable loss of body nitrogen not properly included in the endogenous nitrogen that it is desired to measure. Thomas, and more recently Martin and Robison, have used this method of measuring the endogenous nitrogen excreted in the urine. In applying the values thus determined to the interpretation of balance data obtained in subsequent periods during which diets are consumed containing the protein whose biological value it is desired to measure, an assumption is introduced the justification

for which is purely theoretical. This assumption is to the effect that the catabolism of body substances containing nitrogen, occurring during the feeding of nitrogen-free diets, continues at a constant level when protein feeding is resumed. The assumption conforms with Folin's theory of protein metabolism, but the experimental data of Folin prove simply that the excretion of *creatinine* is constant. Since this substance seems to contain only 20 per cent or less of the urinary nitrogen excreted on a nitrogen-free diet, the theory of a constant endogenous catabolism of nitrogenous substances is not particularly convincing and in many quarters seems to have been discarded entirely.

In another publication (3) the author has discussed critically the theories on this subject, and, on the basis of analyses of the tissues of rats that had subsisted for some considerable time on a nitrogen-free dietary as compared with analyses of the tissues of other rats not so treated, has advanced a theory of endogenous catabolism supplementary to that of Folin and agreeing with it in postulating a constant breakdown of nitrogenous constituents of the tissues regardless of the amount or character of the protein fed. However, the method of measuring the biological value of proteins by the method associated with the name of Thomas, cannot be justified or refuted by theoretical discussion. The question can be settled only by experimental inquiry.

There seems to be no obvious method of settling directly or finally the question whether, during a period of protein feeding, the contribution of the tissues to the urinary nitrogen is the same as the total urinary nitrogen excreted on a protein-free ration; nor on more careful consideration has such a method occurred to the author. It is possible, nevertheless, to throw some light on this question in a more or less indirect way.

If the theory of Sherman as to the nature of the endogenous catabolism (4) is correct, or if the views of Osborne and Mendel (5) conform with the facts, then no constant basal catabolism of body tissue exists. According to these theories, the endogenous catabolism would be largely or entirely suppressed when an animal is receiving protein in its food, either because of a retardation of the hydrolysis of tissue protein caused by the mass action of the amino-acids coming from the digestive tract, or because of the complete suppression of the hydrolysis of tissue protein,

initiated, in the absence of amino-acids of dietary origin, by the urgent need of the body for certain nitrogenous hormones; with the influx of amino-acids from the intestinal tract, exogenous precursors of these hormones become available, and the endogenous catabolism is reduced to the inevitable destruction of the hormones themselves in the course of general metabolism. It may be expected, therefore, if the endogenous catabolism is so readily diminished by protein feeding, that the urinary nitrogen excreted on a diet containing a small amount of a protein of high biological value would be considerably less than the urinary nitrogen excreted on a nitrogen-free diet.

In order to submit this supposition to an experimental test, five rats were put upon a ration of very low nitrogen content (0.06 per cent). On the 5th and 6th days of such feeding, when it may be supposed that the endogenous level of nitrogenous metabolism had been reached (3) daily collections of urine and feces were made and analyzed for nitrogen separately. For the next 3 days of the experiment, the rats were given a ration containing about 2.5 per cent of milk protein (0.399 per cent of nitrogen). This period was followed by a 3 day period on the low nitrogen ration, a 3 day period on a ration containing about 3.0 per cent of lactalbumin (0.506 per cent of nitrogen), and finally a 3 day period on the low nitrogen ration. Daily collections and analyses of urine and feces were made throughout the experiment. The detailed data for two of the rats, which consumed the rations the most satisfactorily, are given in Table X, while the averages for all five rats are summarized in Table XI. The rats were litter mates and weighed very nearly the same.

The excretion of nitrogen in the urine during the 3 days on the milk protein ration was at the same level as the excretion of nitrogen on the last 2 days of the first period of feeding on a low nitrogen ration, and somewhat greater than the level of excretion during the second period of such feeding. However, during the period of lactalbumin feeding, the excretion of urinary nitrogen was slightly though distinctly lower than that in either the preceding or the following period of non-protein feeding.

Following the experimental feeding periods described above, a ration containing a little less than 6 per cent of lactalbumin (0.928 per cent of nitrogen) was offered the five rats, but their

### *Effect of the Ingestion of Small Amounts of Milk Proteins and of Lactalbumin on the Endogenous Excretions of Nitrogen.*

[illegible]



condition was so poor, due to the prolonged period of low protein feeding, that successful results were obtained with only one rat, No. 153. These data will be found in Table X. An intake of twice the amount of lactalbumin nitrogen previously given in-

TABLE XI.  
*Average Data for All Five Rats.*

Experimental day	Ration	Daily food consumed.	Daily intake of nitrogen	Daily urinary nitrogen	Daily fecal nitrogen	Body weight of rats
		<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>gm.</i>
5	Low N.	7 6	5	24	15	130
6	" "	8 1	5	23	15	
Average . . .		7 85	5	23 3	15 4	
7	Milk protein.	5 6	22	20	12	128
8	" "	7 6	30	28	14	
9	" "	6 7	27	21	21	
Average . . .		6 65	26 5	23 1	14 9	
10	Low N.	7 1	4	23	18	124
11	" "	6 8	4	19	14	
12	" "	6 6	4	18	15	
Average . . .		6 83	4	19 9	15 7	
13	Lactalbumin.	7 6	38	15	14	122
14	"	6 5	33	14	14	
15	"	6 2	31	15	12	
Average . . .		6 75	34	14 8	13 4	
16	Low N	5 0	3	17	12	120
17	" "	5 3	3	16	10	
18	" "	5 7	3	17	9	
Average . . .		5 37	3	16 0	10 9	

duced a distinct rise in urinary nitrogen; a following period of non-protein feeding also initiated a distinct fall in urinary nitrogen, though the period was too short for the excretion to reach the endogenous level.

The results of this experiment are somewhat difficult of interpretation. Certainly there is no indication that the mixed pro-

teins of milk had any effect on the excretion of nitrogen in the urine on a nitrogen-free diet. However, on the lactalbumin ration, a distinct drop in the excretion of urinary nitrogen occurred, though during the succeeding period of non-protein feeding, the urinary nitrogen rose only slightly on the average, and to an apparently significant extent only with one of the five rats. The data as a whole do not indicate any marked effect of dietary protein on the rate of catabolism of the nitrogenous constituents

TABLE XII

Rat No	Average weight of rat.	Nitrogen content of ration	Food consumed daily.	Daily nitrogen intake	Daily urinary nitrogen	Endogenous nitrogen.	Food nitrogen in urine.	Daily fecal nitrogen	Metabolic nitrogen in feces.	Food nitrogen in feces	Biological value of protein.
	gm.	per cent	gm	mg	mg.	mg.	mg	mg.	mg.	mg	per cent
2	262	0.654	8.70	56.9	48.6	37.7	10.9	27.6	21.7	5.9	79
1	213	0.800	7.50	60.0	40.2	28.6	11.6	23.1	18.3	4.9	79
3	155	1.043	6.00	62.6	34.5	22.2	12.3	18.1	11.3	6.8	78
4	145	1.043	6.00	62.6	33.4	17.4	16.0	19.0	11.3	7.7	71
5	145	1.043	6.00	62.6	32.8	22.0	10.8	20.9	10.7	10.2	79
3	158	0.800	6.00	48.0	33.0	22.2	10.8	17.3	11.3	6.0	74
4	143	0.800	6.00	48.0	32.8	17.4	15.4	18.4	11.3	7.1	62
5	146	0.800	6.00	48.0	30.2	22.0	8.2	18.0	10.7	7.3	80
1	214	0.654	7.50	49.0	39.3	28.6	10.7	22.6	18.3	4.3	76
3	156	0.654	6.00	39.2	31.3	22.2	9.1	15.9	11.3	4.6	74
4	142	0.654	6.00	39.2	30.5	17.4	13.1	16.5	11.3	5.2	61
5	145	0.654	6.00	39.2	33.6	22.0	11.6	17.0	10.7	6.3	65
6	60	1.043	3.60	37.5	19.2	9.3	9.9	11.6	5.5	6.1	69

of the tissues. The diminution in the excretion of urinary nitrogen with succeeding periods of non-protein feeding is another phenomenon that will be taken up later.

A study of the nitrogen excretion of rats differing widely in weight and receiving approximately the same amounts of a constant protein mixture, may be expected to throw considerable light on the correctness of the assumption of a constant basal catabolism of nitrogenous substances. Such an experiment has already been referred to on page 881, part of the data being there utilized in

connection with the estimation of the metabolic nitrogen of the feces. Complete balance data of those periods in which comparable amounts of protein of a definite character (corn 85, tank-age 15) were consumed, are given in Table XII. The food intake of these rats was adjusted to maintenance of body weight for several months previous to the metabolism experiments.

It will be noted in the three sets of data in Table XII, that, within each set, the daily excretion of urinary nitrogen on comparable nitrogen intakes varied with the body weights of the rats, the larger rats having the larger excretions. Assuming that the endogenous excretion of urinary nitrogen is constant for each rat and is equal to the total urinary nitrogen on a nitrogen-free diet, the figures given in Column 7 enable one to compute that fraction of the urinary nitrogen that was of dietary origin. These figures are given in Column 8. They are fairly constant except for the high figure for Rat 4, which throughout the experiment seemed distinctly and consistently less efficient in the utilization of dietary protein in metabolism than its mates. Similarly, the food nitrogen in the feces may be estimated from the total fecal nitrogen and the metabolic fecal nitrogen determined in a period of feeding a nitrogen-free ration (Columns 9, 10, and 11). Finally, the biological value of the protein, Column 12, may be computed according to the following illustration. Rat 2 consumed an average of 56.9 mg. of nitrogen per day in a 7 day collection period. On this diet, the daily excretion of fecal nitrogen was 27.6 mg., but of this nitrogen it is estimated that 21.7 mg. were derived from the body, leaving only 5.9 mg. contained in the indigestible food protein. Hence, the rat was receiving daily  $56.9 - 5.9 = 51.0$  mg. of absorbed nitrogen. During this period, the excretion of urinary nitrogen was 48.6 mg. daily, of which 37.7 mg. were the result of the catabolism of the body's own tissues. Hence, only  $48.6 - 37.7 = 10.9$  mg. of the absorbed dietary nitrogen were wasted in metabolism, and  $51.0 - 10.9 = 40.1$  mg. were retained in the body. The biological value of the protein, therefore, is equal to  $100 \times (40.1 \div 51.0) = 79$ . The constancy of the computed biological values in the first set of data, except for the one low figure for Rat 4, is remarkable, and testifies to the essential accuracy of the assumptions upon which the calculation is based.

In the other two sets of data the agreement among the biological values is not so good, but, considering the low values for Rat 4 as due to an exceptional physiological performance or to an inaccurate determination of endogenous nitrogen, the remaining values, in the light of the experience of this laboratory in work of this type, exhibit a variability not greatly out of the ordinary.

The ability of this method of measuring the biological value of proteins to harmonize results obtained with rats differing widely in size is further illustrated by the data in Table XIII.

With the first group of rats (Nos. 60 to 64, Table XIII), averaging 181 gm. in weight, the urinary nitrogen amounted to 37.3 per cent of the absorbed nitrogen on the milk protein ration, while with the second group of rats (Nos. 65 to 69), averaging only 91 gm. in weight, the urinary nitrogen on the same ration amounted to only 28.6 per cent of the absorbed nitrogen. The discrepancy may be largely accounted for by the fact that with the heavier rats the endogenous nitrogen in the urine amounted to 18.1 per cent of the absorbed nitrogen, while with the lighter rats it amounted to only 12.7 per cent. Thus, the heavier rats excreted 19.2 per cent of the absorbed nitrogen in the urine, while the lighter rats excreted 15.9 per cent.

For the corn protein ration, containing a smaller percentage of protein, the case is more striking. Here the heavier rats averaged 241 gm., and the lighter rats 97 gm. With the former, the urinary nitrogen averaged 82.4 per cent of the absorbed nitrogen, while with the latter it averaged only 66.2 per cent. The discrepancy is practically entirely accounted for by the great difference in the amount of nitrogen excreted in the urine, resulting from tissue catabolism. With the larger rats this amounted to 55.4 per cent of the absorbed nitrogen, while with the smaller it was only 39.5 per cent. The urinary nitrogen of dietary origin was, therefore,  $82.4 - 55.4 = 27.0$  per cent of the absorbed nitrogen for the rats averaging 241 gm. in weight, and  $66.2 - 39.5 = 26.7$  per cent for the rats averaging 97 gm. in weight. The biological value for the two groups of rats thus averaged very nearly the same, 73 and 74 per cent. It would seem that the close agreement revealed by this method of calculation between two sets of data, seemingly so discordant as regards the utilization of the dietary nitrogen,



testifies to the essential accuracy of the assumptions upon which it is based, prominent among which is the assumption that the endogenous catabolism of nitrogen is not affected by the ingestion of protein.

However, the endogenous catabolism of nitrogenous substances is apparently affected by other factors, in the same manner as the "metabolic nitrogen" of the feces. Thus, from the data

TABLE XIV.  
*Excretion of Urinary Nitrogen on a Nitrogen-Free Ration.*

Rat No	Average weight of rat	Average daily food intake	Average daily excretion of nitrogen in the urine	
			Total	Per 100 gm. body weight.
	gm.	gm	mg	mg.
180	154	3 78	35 8	23 9
181	166	5 55	29 8	18 0
182	169	6 65	26 1	15.4
184	157	7 17	32 0	20 5
183	162.	8 11	29 2	18 0
142	147	5 36	34 1	23.2
140	144	5 82	29 8	20 7
141	126	6 57	24 3	19 3
144	169	8 15	35 3	20 9
143	160	8.51	35 2	22 0
129	97	4 33	21 8	22 5
123	98	5 13	26 1	26.6
127	105	5 84	22 7	21 4
122	91	5 90	27.0	29 7
126	98	6 44	24 5	25 0
124	101	8 30	24 8	24 6

given in Table IV, it is evident that an increase in the intake of energy on a "nitrogen-free" ration will lower the excretion of urinary nitrogen provided the former intake is approximately equal to the maintenance requirement of the rat. That there is no general inverse correlation between intake of nitrogen-free food and excretion of urinary nitrogen, however, is indicated by the values obtained on three groups of rats—each group being under observation at the same time and weighing approximately the same. The data are tabulated in Table XIV, the rats in each

group being arranged in order of increasing intake of nitrogen-free food. All collection periods were of 1 week duration.

Apparently, if the rat is receiving slightly above its requirement of energy for maintenance, the excretion of urinary nitrogen on a nitrogen-free ration is unrelated to the intake of food.

From the data in Table XIV it appears that the endogenous catabolism of nitrogen in rats as measured by the urinary nitrogen on a nitrogen-free ration, varies considerably, even when reduced

TABLE XV

*Excretion of Urinary Nitrogen in Adjacent Periods of Non-Protein Feeding.*

Rat No	Average body weight	Length of collection period	Average daily food intake	Average daily urinary nitrogen.	
				Total	Per 100 gm. body weight.
	<i>gm</i>	<i>days</i>	<i>gm</i>	<i>mg.</i>	<i>mg.</i>
9	103	4	4 82	22 1	21 5
	97	7	5 74	22 8	23 5
11	86	4	4 17	19 6	22 8
	82	4	3 90	16 2	19 8
12	104	7	4 85	27 5	26.2
	97	7	5 76	27 7	28 6
15	100	8	4 44	22 3	22 3
	91	7	4 16	16 3	18 1
16	104	7	5 62	14 9	14 3
	97	7	4 37	14 8	15 2
27	168	7	10 89	30 6	15 2
	164	7	7 81	29 3	14 6

to 100 gm. of body weight. Does this variation represent simply experimental error, or is it indicative of actual differences in physiological functioning? If the latter is true, adjacent periods on a nitrogen-free diet should give fairly concordant results. A few such tests have been performed in this laboratory, and the data obtained have all been collected in Table XV. The data of the fecal excretion in these periods have already been presented in Table VIII.

The agreement among corresponding figures in the last column of Table XV seems as satisfactory in most cases as is ordinarily obtained in biological work. Certainly, the high level of endogenous catabolism indicated by the values obtained with Rat 12 as compared with Rats 16 and 27 may be interpreted as a physiological fact, not an experimental aberration. Similarly, the distinction between Rats 9, 11, and 15, and Rats 16 and 27 is shown.

TABLE XVI

*Results Indicating a Constancy in the Excretion of Endogenous Nitrogen in the Urine in Adjacent Periods.*

Rat No	Average weight	Ration	Average daily food intake	Average daily intake of nitrogen	Average daily excretion of urinary nitrogen	
					Total	Per 100 gm body weight
	<i>gm</i>		<i>gm</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
127	105	Non-protein.	5 84	4 6	22 7	21 4
	103	Milk protein	8 01	66 9	22 8	22.1
	106	" "	8 13	67 9	24 5	23 1
128	82	Non-protein.	5 39	4 3	18 4	22 4
	79	Milk protein.	5 27	44 0	20 2	25 6
	79	" "	5 64	47 1	20 3	25 7
129	97	Non-protein.	4 33	3 4	21 8	22 5
	97	Milk protein.	6 86	57 3	20 4	21 0
	99	" "	6 20	51 8	21 5	21.7
130	63	Non-protein.	3 03	2 5	21 9	34 8
	60	Milk protein.	4 17	34 8	23 1	38 5
	60	" "	4 73	39 5	20 5	34 2

The results of an experiment demonstrating further the true significance of differences in the apparent endogenous catabolism of rats are given in Table XVI. The four rats in this experiment were first subjected to a period of non-protein feeding and then to two periods on a ration containing about 5 per cent of milk protein, which, according to all our experimental data, is, at this level, almost if not actually completely utilized in metabolism.



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The figures in the last column of Table XVI are again fairly concordant, and in particular the comparatively high endogenous catabolism of Rat 130 in the period of non-protein feeding is abundantly substantiated by the results of the two subsequent periods.

While in adjacent periods of observation it seems justifiable to assume that the endogenous catabolism of nitrogenous constit-

TABLE XVII  
*Excretion of Endogenous Nitrogen in the Urine in Periods Several Weeks Apart.*

Rat No	Collection Period 1.			Collection Period 6			Collection Period 12		
	Average weight of rat	Daily food intake	Urinary nitrogen per 100 gm body weight	Average weight of rat	Daily food intake.	Urinary nitrogen per 100 gm body weight	Average weight of rat	Daily food intake	Urinary nitrogen per 100 gm body weight.
	gm.	gm	mg	gm	gm	mg	gm	gm.	mg.
180	154	3 78	23 9	172	9 92	15 0	186	7 5	15 6
181	166	5 55	18 0	180	10 00	17 2	202	7 5	15 2
182	169	6 65	15 4	184	9 00	10 7	193	4 8	14 1
183	162	8 11	18 0	176	10 00	15 4	189	7 4	16 7
184	157	7 17	20 5	176	10 00	16 0	195	8 6	15 2

	Collection Period 1			Collection Period 6			Collection Period 8		
	Average weight of rat	Daily food intake	Urinary nitrogen per 100 gm body weight	Average weight of rat	Daily food intake.	Urinary nitrogen per 100 gm body weight	Average weight of rat	Daily food intake	Urinary nitrogen per 100 gm body weight.
	gm.	gm	mg	gm	gm	mg	gm	gm.	mg.
122	91	5 90	29 7				122	6 56	28 8
123	98	5 13	26.6				127	6 10	23 1
124	101	8 30	24 6				110	5 11	23 9
125	74	4 97	28 9				82	5.27	15 4
126	99	6 44	25 0				108	7.17	16 0
127	105	5 84	21 4	120	6 07	29 0			
128	82	5 39	22 4	95	5 36	25 9			
129	97	4 33	22 5	126	4 97	21 0			
130	63	3 03	34 8	73	3 96	23 4			

uents of the tissues remains constant, biologically speaking, and is commensurate with the excretion of urinary nitrogen on a nitrogen-free ration, there is abundant evidence that this type of metabolism varies in the course of more extended periods of time, similar to the basal metabolism of energy and possibly for the same reasons. The data collected in Table XVII indicate such changes.

The rats of the first group (Nos. 180 to 184, Table XVII, inclusive) were fed the same throughout the experiment. However, a lowering of the endogenous catabolism apparently occurred in some of the rats, particularly No. 184, while with the others, approximate constancy obtained. The values for Rat 180 in Period 1 and Rat 182 in Period 12, are possibly too high due to an insufficient food intake.

Occasionally, remarkable changes in the urinary output of nitrogen on a nitrogen-free ration occurred, well illustrated by the data for the second group of rats (Nos. 122 to 130 inclusive), also included in Table XVII. These rats were not all fed the same during the periods intervening between the periods con-

TABLE XVIII.  
*Biological Values Obtained during Periods of Protein Feeding.*

Rat No	Period					
	2	3	4	5	6	7
122	71	64	59	63	81	83
123	73	63	63	64	80	80
124	69	59	37	34	73	68
125	58	56	31	22	56	60
126	70	61	34	31	70	66
127	101	102	94	86		
128	95	96	88	85		
129	101	100	85	86		
130	90	94	86	86		

sidered in the table. The high endogenous catabolism of Rat 122 in Period 1 still persists to Period 8. With Rats 123 and 124 insignificant decreases seem to have occurred in this interval, while with Rats 125 and 126, marked decreases occurred. With the other four rats, two marked increases and one decrease in endogenous catabolism are indicated by the values in the table.

In determining whether the marked variations in endogenous catabolism, indicated for some of the rats in the latter group, are real or not, the consistency of the biological values for the proteins investigated during the intervening periods among those rats fed alike, should constitute good evidence one way or the other.

These values are listed in Table XVIII; those rats fed the same throughout being grouped together.

Rats 122 and 123 gave values throughout agreeing as closely as could be expected. Rats 124 and 126 gave values agreeing very closely throughout, though the values for the former rat were computed on the assumption of a constant endogenous catabolism, and those of the latter rat on the assumption of a marked reduction from 25.0 to 16.0 mg. of nitrogen per 100 gm. of body weight. The values for Rat 125, were lower than those for the other two rats, indicating a markedly less efficient physiological utilization of protein. However, they were consistently lower, in spite of the fact that they were calculated on the assumption of a reduction in endogenous catabolism from 28.9 to 15.4 mg. of nitrogen per 100 gm. of body weight in 6 weeks. If such a reduction had not actually occurred, the computed biological values for Rat 125 would have been much lower as compared with those for Rat 124 during Periods 6 and 7 than during Periods 2 and 3. On the other hand, if the biological values in Period 7 are computed on the basis of the endogenous catabolism as measured in Period 1, the results would be 69, 74, and 73, instead of 68, 60, and 66. The former values indicate a superior efficiency of Rat 125 over the other two rats, quite contradictory to the results in Period 2. The evidence with these three rats seems to be a clear substantiation of the theory upon which the calculation of these biological values is based.

The biological values for the last four rats were obtained in four periods in which milk protein was fed at two levels, approximately 5 per cent in Periods 2 and 3, and approximately 10 per cent in Periods 4 and 5. For the latter two periods good agreement exists among the values obtained with one exception; in the former periods the agreement was not so good, though with the smaller amounts of nitrogen contained in both food and excretions, greater variability among the resulting biological values is to be expected due to an accentuation of unavoidable experimental errors. Rats 127 and 129 in all but one period gave biological values agreeing well, though, in one case the assumption was that a marked increase in endogenous catabolism occurred from 21.4 to 29.0 mg. of nitrogen per 100 gm. of weight, while in the latter case a constant catabolism was assumed. Simi-

larly, Rats 128 and 130 gave values agreeing well throughout, though the assumptions involved a slight increase in one case and a marked decrease in the other in the endogenous catabolism. Recalculating the biological values of Period 5, using the values for the endogenous catabolism of nitrogen obtained in Period 1, gives the following results, respectively: 79, 82, 87, and 93.

In view of such variations in the intensity of those physiological processes involved in the degradation of nitrogenous constituents of the tissues, we have modified our experimental procedure in metabolism studies in rats, by inserting periods of non-protein feeding every 4 to 6 weeks throughout a series of studies on the biological value of proteins, and assuming, where the urinary nitrogen per unit of weight on nitrogen-free feeding varies significantly from one such period to the other, that in the intervening periods the endogenous catabolism varies in a regular fashion from week to week. It is also advisable not to use one group of rats for a very extended series of metabolism studies, since the conditions imposed seem to undermine the conditions of the rats as reflected in progressively diminishing appetite. Very probably the administration of a vitamin B preparation separately from the ration, as recommended by Osborne and Mendel, would aid in maintaining a satisfactory food intake throughout a series of metabolism tests.

#### SUMMARY.

A method of measuring the biological value of protein is described and submitted to extensive investigation. The method is based upon nitrogen balance data obtained under definite experimental conditions, and involves direct determinations of the amount of nitrogen in the feces and in the urine and indirect determinations of the fractions of the fecal nitrogen and of the urinary nitrogen that were of dietary origin. The biological value of the protein is taken as the percentage of the absorbed nitrogen (nitrogen intake minus fecal nitrogen of dietary origin) that is not eliminated in the urine. This value is similar to that of Thomas though the reasoning upon which it is based seems more direct, and in particular does not involve the question of the precise significance of the so called "metabolic nitrogen" of the feces.

The experimental investigations, bearing on the validity of the method of calculation used, seem to justify the following conclusions.

1. It is possible to secure reliable data on the nitrogenous metabolism of rats.

2. The "metabolic nitrogen" of the feces on a protein-containing diet is related to the amount of food consumed, and may be measured by the total excretion of fecal nitrogen on a nitrogen-free diet. The latter may be used with the most confidence when the "roughage" content of the nitrogen-free diet approximates that of the protein-containing diet.

( 3. The excretion of nitrogen in the urine resulting from the catabolism of the tissues is not markedly depressed, if at all, by protein feeding. There seems to be a true basal catabolism of nitrogenous substances in the tissues, such that the amount of nitrogen of body origin excreted in the urine when an animal is on a protein-containing dietary may be satisfactorily measured by the total excretion of nitrogen in the urine in an adjacent period of feeding a nitrogen-free diet.) While this endogenous catabolism of nitrogen does not seem to be affected by an influx of amino-acids from the intestinal tract, it may vary markedly in the course of a protracted series of metabolism experiments for unknown reasons, so that in such a series of experiments it is essential to redetermine the intensity of the endogenous catabolism by periods in which nitrogen-free diets are fed, if the most reliable biological values for protein are desired.

The biological value of the protein of a food applied to its content of digestible protein has much the same significance as the coefficient of digestibility applied to the content of total protein. The latter operation gives the content of digestible protein in the food, and this multiplied by the biological value and divided by 100 gives what the author has elsewhere termed (6) the net protein value of the food. Admittedly the net protein value of a food is not necessarily constant, any more than its content of digestible protein. There are many factors modifying the digestibility of the proteins of a food as well as the biological value of the digestible fraction, and the net protein content will be subject to the influence of all such factors. The net protein conception, however, may prove to be of value in determining whether

a given ration or diet should satisfy a given protein requirement, or in compounding a ration or diet that should in the most satisfactory manner cover the protein requirement of an animal under specified conditions. In this respect it is entirely analogous to the net energy conception of Armsby, which has been of such great value in the expression of the actual energy value of farm feeds as related to the energy requirements of farm animals.

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## THE BIOLOGICAL VALUE OF PROTEINS AT DIFFERENT LEVELS OF INTAKE.

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The biological value of a protein, as defined and measured by the method developed in the preceding paper, can hardly be expected to remain constant under all conditions. It may be expected to vary with the use to which the protein is put in the body. The maintenance of the nitrogenous integrity of the tissues, the synthesis of new tissue in growth, and the synthesis of milk proteins in lactation may be expected to require different proportions of amino-acids, so that the nitrogen of a given protein may be expected to be more completely utilized in serving some one of these functions than in serving another. These differences in biological value are concerned with the amino-acid make-up of the protein as compared with the several distinct requirements for amino-acids.

It is generally believed, in fact, that the chemical constitution of a protein determines entirely its biological value. This belief tacitly assumes that the economy with which the cells utilize the amino-acids supplied to them is unaffected by the abundance of the supply, particularly by the rate at which the amino-acids are transported to the cells from the intestinal tract. It also assumes that the undigested fraction of a dietary protein is representative in its amino-acid make-up of the protein as consumed, so that factors influencing the digestibility of the protein would have no effect on the proportions of the different amino-acids absorbed into the blood. It must also assume that no great differences in the rate at which different amino-acids are liberated from a protein during digestion occur, or that if such differences do occur, the storage capacity of the tissues for absorbed amino-acids is sufficiently great to insure the presence there throughout the digestive period of optimum proportions for synthetic purposes of the several



amino-acids. It also assumes that until the demands of the body for protein are covered by the protein intake, amino-acids are not oxidized in the body except in so far as they are unavailable for synthetic purposes, and hence that factors affecting the intensity of the oxidation processes can have no effect on the utilization of amino-acids for structural purposes. These assumptions are either extremely improbable or highly debatable.

However, by conducting experiments with different proteins under the same experimental conditions, particularly with regard to the concentration of protein in the ration, it should be possible to obtain biological values indicating the relative utilization of the proteins in anabolism, and by changing experimental conditions, it should be possible to determine to what extent the assumptions enumerated in the preceding paragraph deviate from the truth.

With these purposes in mind a large number of metabolism studies on rats were undertaken in which a considerable variety of proteins was tested at different levels of intake. The methods of confining the rats and of making the collections of urine and feces have already been described in the preceding paper. The collection periods were all of 1 week duration and were always preceded by a period of at least 3 days in length in which the same ration in approximately the same amounts was fed. Each rat used in these experiments was subjected to at least one period of non-protein feeding to determine the rate of the endogenous catabolism of nitrogenous substances as related to body weight, and the rate of endogenous losses of nitrogen through the digestive tract as related to the amount of food consumed. The values thus obtained were used in the estimation in periods of protein feeding, of the contribution by the body to the fecal and urinary nitrogen and, by difference, the waste of food nitrogen in digestion and metabolism. The methods by which these estimates were made and their justification have already been fully discussed. The biological value of the protein, or more correctly of the total nitrogen in the ration, is finally expressed as the percentage of the absorbed nitrogen which is retained in the body.

In the first series of experiments the proteins or mixtures of proteins tested constituted approximately 5 per cent of the rations fed, all of which possessed approximately the same energy value. The results of these experiments are summarized in Tables I to VII inclusive.

The mixed proteins of milk evidently gave the highest biological values of all the proteins tested, averaging 93.4. Some preliminary tests have been run on the proteins of meat, indicating that, under optimum conditions, meat proteins separated from the extractives are fully as completely utilized as the proteins of milk. If the

TABLE I.  
*Biological Value of Milk Proteins at a 5 Per Cent Level.\**

Rat No	Initial weight <sup>f</sup>	Final weight	Food intake	Nitrogen intake	Fecal nitrogen	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine	Absorbed nitrogen retained in body	Biological value
	gm	gm	gm	mg	mg	mg.	mg	mg	mg	mg	per cent
65	70	80	7 67	63 1	11 1	0 0	63 1	15 4	3 8	59 3	94
	80	83	6 79	55 9	13 0	2 3	53 6	18 8	6 7	46 9	87
66	52	62	6 07	50 0	15 0	4 5	45 5	10 8	0 8	44 7	98
	62	65	6 00	49 4	12 0	1 2	48 2	14 1	2 8	45 4	94
67	63	70	5 81	47 8	11 6	1 8	46 0	13 3	3 9	42 1	92
	70	70	4 90	40 3	11 2	2 5	37 8	17 1	7 1	30 7	81
68	59	68	7 07	58 2	10 4	0 0	58 2	15 8	4 9	53 3	92
	68	74	7 00	57 6	13 7	0 0	57 6	19 5	7 4	50 2	87
69	60	66	6 29	51 8	11 5	0 1	51 7	22 0	7 8	43 9	85
	66	70	6 00	49 4	12 6	1 2	48 2	17 2	2 1	46 1	96
127	101	105	8 01	66 9	20 7	2 1	64 8	22 8	0 0	64 8	100
	105	107	8 13	67 9	31 1	12 2	55 7	24 5	0 0	55 7	100
128	79	80	5 27	44 0	13 7	3 2	40 8	20 2	2 0	38 8	95
	80	79	5 64	47 1	22 0	9 7	37 4	20 3	1 5	35 9	96
129	94	100	6 86	57 3	15 3	0 0	57 3	20 4	0 0	57 3	100
	100	99	6 20	51 8	24 6	7 3	44 5	21 5	0 0	44 5	100
130	61	60	4 17	34 8	15 8	0 0	34 8	23 1	3 6	31 2	90
	60	60	4 73	39 5	19 6	1 2	38 3	20 5	2 4	35 9	94

Average biological value . . . . . 93 4

\* Results in this and the following tables expressed on the daily basis.

extractives are not removed, however, the average biological value of the total nitrogen of meat is distinctly less than that of milk, probably due to a very low biological value of the nitrogenous extractives. These results on meat are not sufficiently numerous to warrant reporting at this time.

The main protein of milk, as other investigations have shown, is distinctly less efficient in nutrition than the mixed proteins of milk. The average biological value obtained for casein was 70.8. At a 5 per cent level of intake, casein, in fact, seems to be no higher in biological value than the proteins of corn, for which an average value of 72.0 was obtained.

TABLE II  
*Biological Value of Casein at a 5 Per Cent Level.*

Rat No	Initial weight	Final weight	Food intake	Nitrogen intake	Fecal nitrogen	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine	Absorbed nitrogen retained in body	Biological value
	gm	gm	gm	mg	mg	mg	mg	mg	mg	mg	per cent
20	61	65	4 61	45 6	11 5	3 5	42 1	28 8	14 4	27 7	66
21	72	76	4 91	48 2	11 4	3 0	45 2	30 1	15 6	29 6	66
22	64	64	3 86	36 1	8 2	1 6	34 5	24 2	9 7	24 8	72
	64	64	3 87	36 5	6 8	0 2	36 3	24 9	10 4	25 9	71
23	81	80	3 73	34 8	8 2	0 5	34 3	27 7	9 1	25 2	73
	80	81	4 73	44 7	9 2	0 0	44 7	32 0	13 4	31 3	70
27	144	149	7 67	70 1	21 4	8 9	61 2	43 5	17 2	44 0	71
	149	165	10 56	96 5	23 0	5 8	90 7	50 3	21 7	69 0	76
15	113	119	9 44	86 3	20 3	1 8	84 5	50 0	26 6	57 9	69
	118	124	9 09	80 1	24 1	6 3	73 8	43 0	18 6	55 2	75
16	110	120	9 09	83 1	23 2	6 2	76 9	33 8	16 9	60 0	72
65	58	64	6 16	54 3	8 9	0 0	54 3	23 6	13 8	40 5	75
66	45	47	3 84	33 9	6 1	0 0	33 9	17 7	10 0	23 9	71
67	56	59	5 11	45 1	8 2	0 6	44 5	20 8	12 6	31 9	72
68	56	58	4 50	39 7	9 0	2 0	37 7	22 9	12 7	25 0	66
69	56	56	4 94	43 6	7 3	0 0	43 6	27 0	14 1	29 5	68
Average biological value . . . . .											70 8

The biological values of the mixed proteins of the three cereals examined, corn, oats, and whole rice, show rather distinct differences, the average values being 72.0 for corn, 78.6 for oats, and 86.1 for rice at a 5 per cent level. These differences seem to be fairly distinct. Of the four rats on which tests both of corn and oat proteins were run, three gave distinctly higher values for oat protein than for corn protein, while one gave concordant

values for both cereals. Of the two rats on which tests both of oat and of rice proteins were made, both gave higher values for rice proteins.

TABLE III.  
*Biological Value of Corn Proteins at a 5 Per Cent Level.*

Rat No	Initial weight	Final weight	Food intake	Nitrogen intake.	Fecal nitrogen	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine	Absorbed nitrogen retained in body	Biological value.
	gm.	gm	gm	mg	mg	mg	mg	mg	mg	mg.	per cent
60	232	230	10 94	87 8	29 3	8 1	79 7	65 2	23 9	55 8	70
61	250	249	10 10	81 1	27 0	8 3	72 8	63 3	18 0	54 8	75
	249	241	9 23	74 1	26 4	9 3	64 8	60 2	15 6	49 2	76
62	244	245	10 56	84 8	27 4	10 3	74 5	63 5	18 2	56 3	76
63	240	243	11 03	88 6	30 1	11 1	77 5	59 1	22 2	55 3	71
65	115	109	5 94	47 7	13 6	5 2	42 5	30 9	12 6	29 9	70
	109	108	5 54	44 5	10 8	1 4	43 1	28 2	12 9	30 2	70
66	93	88	5 66	45 4	10 5	0 0	45 4	28 1	11 2	34 2	75
	88	88	5 60	45 0	11 3	0 0	45 0	26 8	9 9	35 1	78
67	100	98	5 83	46 8	13 3	1 8	45 0	27 8	13 6	31 4	70
	98	98	4 67	37 5	9 4	0 0	37 5	22 9	8 9	28 6	76
68	105	98	6 19	49 7	16 7	2 8	46 9	35 0	18 3	28 6	61
	98	94	4 97	39 9	12 1	0 2	39 7	27 2	11 7	28 0	70
69	98	98	6 67	53 6	15 6	1 9	51 7	40 1	18 9	32 8	63
	98	95	6 83	54 8	15 4	0 8	54 0	33 2	12 4	41 6	77
15	111	109	8 29	75 0	20 5	4 3	70 7	39 8	17 6	53 1	75
	109	110	8 49	75 5	20 5	3 9	71 6	39 7	17 7	53 9	75
16	109	106	7 47	67 6	18 5	4 5	63 1	39 4	23 7	39 4	62
	106	106	8 11	72 1	16 6	1 4	70 7	39 5	23 9	46 8	66
20	69	72	5 33	49 4	13 2	3 9	45 5	25 9	9 9	35 6	78
	72	72	5 23	47 3	13 6	4 5	42 8	26 2	9 8	33 0	77
21	87	88	5 74	53 2	15 3	5 4	47 8	29 2	12 1	35 7	75
	88	87	6 20	56 1	14 4	3 7	52 4	32 3	15 2	37 2	71
Average biological value..											72

The proteins of yeast, at this low level of intake, also seem to be very completely utilized, as indicated by an average biological value of 85.5. The average biological value for potatoes, 68.5, was the

TABLE IV.

*Biological Value of Oat Protein at a 5 Per Cent Level.*

Rat No.	Initial weight.	Final weight	Food intake	Nitrogen intake.	Fecal nitrogen.	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine	Absorbed nitrogen retained in body.	Biological value.
	gm.	gm	gm	mg.	mg	mg	mg	mg	mg.	mg.	per cent
15	96	100	6 83	60 7	19 7	6 3	54 4	30.4	10 6	43 8	80
	100	105	7 30	64 9	18.7	4 4	60 5	31.0	10 4	50 1	83
16	94	96	5 29	47 0	14 6	4 8	42 2	24 9	11 0	31 2	74
	96	99	6 54	58 1	17 8	5 6	52 5	28 2	13 9	38 6	74
20	70	70	5 35	47 6	13 9	4 6	43 0	25 1	9 1	33 9	79
	70	77	6.36	55.6	15 4	4 3	51 3	27.3	10 7	40 6	79
21	85	85	5 87	52 2	14 8	4.7	47 5	26 0	9 3	38 2	80
	85	90	6 49	56 8	16 4	5 2	51 6	27 2	10.1	41 5	80

Average biological value . . . . . 78.6

TABLE V.

*Biological Value of Rice Protein at a 5 Per Cent Level.*

Rat No.	Initial weight.	Final weight	Food intake	Nitrogen intake	Fecal nitrogen	Food nitrogen in feces.	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine.	Absorbed nitrogen retained in body.	Biological value.
	gm.	gm	gm	mg.	mg	mg	mg.	mg	mg	mg.	per cent
22	68	76	7.79	67 6	14 9	1 7	65 9	25 6	9 3	56 6	86
23	90	94	7 64	66 3	17.1	1 4	64 9	28 5	7 2	57 7	89
24	92	93	6 96	60 4	15.1	3 6	56 8	30.1	9 4	47.4	83
25	56	58	5 29	45 9	12 1	2 8	43 1	22 8	5 2	37.9	88
20	79	82	6 37	55.4	12 4	1 3	54 1	26 6	8 4	45 7	84
	82	87	6 04	52.5	11 7	1 2	51 3	25 2	6.0	45 3	88
21	95	101	8 21	71 3	16 4	2 3	69 0	31.9	12 7	56 3	82
	101	108	7 13	62 0	15 3	3.0	59.0	26.9	6 5	52.5	89

Average biological value..... 86.1

TABLE VI.

*Biological Value of Potato Nitrogen at a 5 Per Cent Level.*

Rat No.	Initial weight.	Final weight.	Food intake	Nitrogen intake	Fecal nitrogen	Food nitrogen in feces.	Absorbed nitrogen	Urinary nitrogen.	Food nitrogen in urine.	Absorbed nitrogen retained in body	Biological value.
	gm.	gm	gm	mg	mg	mg	mg	mg	mg	mg.	per cent
140	140	141	9 41	82 2	40 2	21 7	60 5	45 6	18 2	42 3	70
	141	144	9 04	79 0	35 2	18 0	61 0	44 0	17 6	43 4	71
141	126	125	8 56	74 8	28 5	16 9	57 9	39 8	17 2	40 7	70
	125	127	8 23	71 9	26 6	15 7	56 2	39 9	18 5	37 7	67
142	140	140	8 32	72 7	22 3	9 3	63 4	46 1	16 1	47 3	75
	140	141	8 97	78 4	30 7	17 4	61 0	46 6	19 2	41 8	68
143	153	154	11 82	103 3	24 1	0 9	102 4	55 6	23 9	78 5	77
	154	152	9 84	86 0	36 9	19 1	66 9	54 5	24 8	42 1	63
144	161	156	8 48	74 1	29 3	15 7	58 4	53 2	21 0	37 4	64
	156	159	9 70	84 8	37 6	23 3	61 5	55 8	24 7	36 8	60

Average biological value. . . . . 68 5

TABLE VII

*Biological Value of Yeast Nitrogen at a 5 Per Cent Level.*

Rat No	Initial weight.	Final weight.	Food intake	Nitrogen intake	Fecal nitrogen.	Food nitrogen in feces.	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine.	Absorbed nitrogen retained in body	Biological value.
	gm	gm	gm	mg	mg	mg	mg.	mg	mg	mg	per cent
24	86	87	6 16	57 3	25 1	14 9	42 4	26 9	7 6	34 8	82
	87	88	6 10	56 7	24 1	14 0	42 7	25 1	5 5	37 2	87
	88	89	6 34	59 0	26 2	15 7	43 3	24 7	4 9	38 4	89
25	53	52	5 10	47 5	20 5	11 5	36 0	22 4	6 4	29 6	82
	52	52	4 81	44 7	21 8	13 3	31 4	20 2	4 2	27 2	87
	52	51	4 54	42 3	18 4	10 4	31 9	20 1	4 4	27 5	86

Average biological value..... 85.5

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lowest of any thus far considered; the individual values obtained in the potato tests were, however, not particularly uniform. It should be mentioned in connection both with the yeast and the

TABLE VIII  
*Biological Value of Milk Proteins at a 10 Per Cent Level.*

Rat No	Initial weight	Final weight	Food intake	Nitrogen intake	Fecal nitrogen	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine.	Absorbed nitrogen retained in body	Biological value.
	gm.	gm	gm	mg	mg	mg	mg	mg.	mg.	mg	per cent
65	91	109	8 00	128 5	17 1	4 1	124 4	33 9	19 1	105 3	85
	123	141	8 86	142 3	22 1	6 5	135 8	39 7	21 4	114 4	84
66	74	91	8 00	128 5	27 2	12 2	116 3	32 2	17 2	99 1	85
	98	117	8 86	142 3	26 8	8 3	134 0	38 1	17 4	116 6	87
67	82	99	8 00	128 5	20 1	5 1	123 4	29 5	16 6	106 8	86
	109	131	8 86	142 3	22 5	3 2	139 1	36 0	18 7	120 4	87
68	83	101	8 00	128 5	22 3	5 4	123 1	38 5	23 1	100 0	81
	106	127	8 86	142 3	24 2	1 8	140 5	41 7	23 4	117 1	83
69	82	96	8 00	128 5	23 1	7 3	121 2	40 0	20 5	100 7	83
	107	124	8 86	142 3	28 6	8 9	133 4	43 0	18 7	114 7	86
127	117	132	9 70	144 1	49 2	26 7	117 4	39 0	6 8	110 6	94
	132	140	9 57	142 2	33 1	10 9	131 3	55 5	18 1	113 2	86
128	82	98	7 11	105 7	32 7	15 9	89 8	32 4	10 4	79 4	88
	98	110	7 69	114 3	42 4	22 7	91 6	39 7	13 5	78 1	85
129	112	131	10 31	153 2	52 2	23 4	129 8	46 1	19 8	110 0	85
	131	146	11 01	163 6	38 0	7 3	156 3	51 4	21 5	134 8	86
130	64	76	5 26	78 2	29 1	8 6	69 6	29 6	10 0	59 6	86
	76	85	6 53	97 0	22 4	0 0	97 0	34 5	13 9	83 1	86
60	164	183	11 70	187 2	46 0	17 5	169 7	65 0	29 2	140 5	82
61	176	197	11 93	190 9	52 2	22 6	168 3	55 2	28 2	140 1	84
62	184	199	11 77	188 3	48 2	18 2	170 1	64 1	35 3	134 8	80
63	183	195	11 11	177 8	45 0	18 1	159 7	66 2	38 0	121 7	76
64	156	176	10 90	174 4	39 2	10 3	164 1	58 0	29 3	134 8	82

Average biological value

84.7

potato, that the biological values obtained, being based entirely on nitrogen balance studies, do not even approximately apply to the *proteins* of these foods, since so much non-protein nitrogenous material is contained in them. With most foods, the biological

value of the absorbed nitrogen, can within small limits of error be taken as representative of the biological value of their mixed proteins.

The second series of experiments was performed on rations containing approximately 10 per cent of protein ( $N \times 6.25$ ). While

TABLE IX  
*Biological Value of the Proteins of Corn at a 10 Per Cent Level.*

Rat No	Initial weight	Final weight	Food intake	Nitrogen intake	Fecal nitrogen	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine	Absorbed nitrogen retained in body	Biological value.
	gm	gm	gm	mg	mg	mg	mg	mg	mg	mg	per cent
65	131	131	5 27	82 3	16 3	6 0	76 3	51 2	31 8	44 5	58
	146	147	7 04	110 2	21 2	5 4	104 8	72 4	47 4	57 4	55
66	103	102	4 20	65 6	13 4	4 4	61 2	44 8	22 6	38 6	63
	119	121	6 66	104 3	19 9	5 6	98 7	64 5	34 4	64 3	65
67	120	119	4 59	71 7	14 1	3 9	67 8	44 1	25 3	42 5	62
	141	142	6 47	101 3	20 4	6 0	95 3	64 7	38 3	57 0	60
68	113	111	4 89	76 4	18 8	6 0	70 4	44 6	25 7	44 7	63
	130	134	7 31	114 5	23 1	3 9	110 6	67 2	41 7	68 9	62
69	110	106	4 80	75 0	17 3	6 7	68 3	48 2	24 7	43 6	64
	120	120	6 36	99 6	18 4	5 7	93 9	64 0	36 4	57 5	61
60	222	223	10 66	166 9	33 9	13 2	153 7	122 4	82 7	71 0	46
	223	223	8 79	137 7	30 6	13 6	124 1	106 3	66 4	57 7	46
61	241	242	9 56	149 7	28 6	10 9	138 8	112 3	68 4	70 4	51
	242	244	9 23	144 5	28 2	11 1	133 4	102 1	57 9	75 5	57
62	245	250	11 46	179 5	35 1	16 4	162 1	123 5	77 8	85 3	52
	250	250	9 21	144 7	33 6	18 6	126 1	95 5	49 3	76 8	61
190	202	213	10 00	136 5	20 9	2 4	134 1	81 3	48 6	85 5	64
191	201	212	10 00	136 5	23 0	0 3	136 2	74 3	45 3	90 9	67
192	207	218	10 00	136 5	21 2	0 8	135 7	80 9	42 3	93 4	69
193	204	220	10 00	136 5	22 4	3 0	133 5	82 6	41 9	91 6	69
194	208	220	10 00	136 5	23 8	7 8	128 7	81 4	48 0	80 7	63

Average biological value . . . . . 59.6

in the former series, the dietary protein was used mainly for the maintenance of the tissues, in this series some growth was also obtained. The experimental results are summarized in Tables VIII to XI, inclusive.



TABLE X.

*Biological Value of Oat Proteins at a 10 Per Cent Level.*

Rat No.	Initial weight.	Final weight	Food intake.	Nitrogen intake	Fecal nitrogen	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine	Absorbed nitrogen retained in body	Biological value
	gm	gm	gm	mg	mg	mg	mg	mg.	mg	mg	per cent
60	199	209	13 57	219 2	61 5	31 2	188 0	88 8	49 0	139 0	74
	209	210	12 77	206 2	49 9	22 3	183 9	94 6	54 7	129 2	70
61	211	221	14 54	234 8	60 8	28 7	206 1	100 0	65 2	140 9	68
	221	227	15 16	244 8	66 1	34 0	210 8	113 7	76 5	134 3	64
62	212	223	13 80	222 9	55 9	26 1	196 8	103 4	67 4	129 4	66
	223	229	13 83	223 4	56 1	28 0	195 4	103 5	64 7	130 7	67
63	211	221	13 46	217 4	58 7	30 2	187 2	106 3	73 7	113 5	61
	221	222	12 94	209 0	56 4	30 3	178 7	113 4	80 0	98 7	55
64	200	203	13 33	215 3	64 6	31 3	184 0	107 2	71 0	113 0	61
	203	205	12 53	202 4	52 9	22 2	180 2	104 4	67 1	113 1	63

Average biological value. . . . . 64.9

TABLE XI

*Biological Value of Potato Proteins ( $N \times 6.25$ ) at a 10 Per Cent Level.*

Rat No.	Initial weight	Final weight	Food intake	Nitrogen intake	Fecal nitrogen	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine.	Absorbed nitrogen retained in body	Biological value.
	gm	gm	gm.	mg	mg.	mg	mg	mg.	mg	mg	per cent
140	157	164	9 41	119 2	31 7	15 8	103 4	58 9	34 4	69 0	68
	164	160	7 65	96 9	19 2	6 8	90 1	54 4	31 2	58 9	65
141	139	149	10 00	126 7	27 5	15 1	111 6	58 5	39 1	72 5	65
	149	164	11 60	147 0	26 0	12 0	135 0	68 4	49 2	85 8	64
142	154	165	10 00	126 7	20 1	7.7	119 0	58 8	36 2	82 8	70
	165	179	11 60	147.0	30 0	16 6	130 4	63 5	42 2	88 2	68
143	165	172	10 00	126 7	27 7	14 1	112 6	59 2	33 2	79 4	71
	172	189	11 60	147 0	33 7	19 7	127 3	64 2	38 6	88 7	70
144	166	170	9 19	116.4	24 6	14 7	101 7	66 0	35 3	66 4	65
	170	171	10 00	126 7	24 3	14 8	111 9	73 7	43 6	68.3	61

Average biological value..... 66.7

As might be expected, the increase in the level of protein feeding has lowered the biological value of the protein. With the mixed proteins of milk this decrease is not great, the biological value falling from 93.4 at the 5 per cent level to 84.7 at the 10 per cent level. It seems, however, to be distinct. Comparing the values for Rats 65 to 69, inclusive, at both levels (Tables I and VIII), it will be seen that four of these five rats gave distinctly lower values at the higher level. Rat 67 gave such discordant values at the 5 per cent level, that no conclusion one way or the other is justified in this case.

Corn proteins show a more distinct drop in biological value, from 72.0 to 59.6. The reality of such a reduction in value is strengthened by the fact that eight of the rats receiving the 10 per cent ration (Table IX) had in closely adjacent periods received a 5 per cent ration of corn proteins (Table III) and in each case the experimental results indicated a distinct drop in the biological value of the protein. The lowest values at the 10 per cent level were obtained with a group of four rats weighing close to 250 gm. Possibly these exceptionally low values were due in part to an excess of protein in the diet relative to the protein requirements of the animals. Against this possibility may be cited the results obtained by Nevens working in this laboratory (1) on a group of three rats weighing 108 to 118 gm. On a ration containing approximately 10 per cent of corn protein, biological values of 48, 49, and 52 were obtained. On the other hand, in another investigation in this laboratory (2) an average biological value of 72 was obtained on three rats on a 5 per cent ration of corn protein, checking exactly the average value for Table III. Occasionally, due either to differences in the physiological economy of rats or to differences in the character of the protein in a given food, such discordant values are obtained.

With oat proteins also, a drop in biological value with an increase in the protein concentration of the ration occurred, from 78.6 (Table IV) to 64.9 (Table X). The superiority in biological value of oat proteins over corn proteins seems just as marked at the higher level as at the lower level. This is particularly clear on comparing the data for Rats 60, 61, and 62, on which both foods were tested.

On the other hand, the nitrogen of potato shows practically the same biological value at a 10 per cent as at a 5 per cent level of intake, the average for the former being 66.7 (Table XI), and for the latter 68.5 (Table VI). The same group of five rats was fed both rations in adjacent periods.

In a previous publication from this laboratory already referred to (2) data were obtained indicating a marked decrease in the biological value of the proteins of the coconut from an average of 77 for a 5 per cent ration to an average of 58 for a 10 per cent ration. In a report on the growth-promoting value of lactalbumin and of casein, Osborne, Mendel, and Ferry have shown that, with rats fed rations in which the amount of these proteins was the only factor limiting growth, the gains in weight per gram of protein consumed, increased to a maximum and then decreased rapidly as the protein concentration of the ration increased continuously (3). If these data are recalculated, using data previously reported by the same investigators on the maintenance requirement of rats for lactalbumin and casein, to obtain the gains in weight *per gram of protein consumed above maintenance*, a continuous decrease in this value is shown for lactalbumin as the protein concentration of the ration is increased. For casein a similar decrease was obtained except for the ration containing the lowest percentage of this protein. In a later report on the nutritive value of the cereal proteins at different levels of intake (4), similar results were obtained. These experimental results confirm our results in indicating a reduction in the biological value of proteins as their concentration in the ration is increased.

It is noteworthy that this decrease in biological value of the proteins of foods is related to the protein concentration of the ration, not to the protein intake. For example, in Tables II, III, and VIII, particularly, marked differences in nitrogen intake, due to a varying consumption of rations containing the same concentration of a given protein mixture, seem to have no effect on the biological value of the protein.

The decrease in the utilization of protein in anabolism as the level of intake increases is probably due in part to a lower utilization for growth than for maintenance and to the increasing ratio of the absorbed nitrogen used for growth to that used for maintenance as the rate of growth increases. However, there is

reason for supposing that this factor cannot account for all of this decrease. Our data on this point are incomplete, probably only indicative. For example, we have found that milk proteins consumed in amounts comparable to the endogenous catabolism of nitrogen have apparently a biological value of 100, since no increase in the urinary nitrogen occurs above the excretion on a nitrogen-free diet (see preceding paper, Table XI). We may, therefore, assume that in covering the maintenance requirement, at this level of intake at least, these proteins are completely utilized in metabolism. It is interesting, therefore, to analyze the balance data obtained on feeding these proteins at higher levels. We may utilize the average data for Rats 65 to 69, inclusive, given in Tables I and VIII. On a ration containing approximately 5 per cent of milk proteins these rats consumed on an average 51.5 mg. of absorbed nitrogen per day, using 90.7 per cent of this amount for maintenance and growth. Estimating the maintenance requirement from the excretion of urinary nitrogen on a nitrogen-free diet, 11.7 mg. were used for this purpose and 34.6 mg. were used for growth. Assuming that the utilization of milk nitrogen at this level is still 100, it is evident that  $51.5 - 11.7 = 39.8$  mg. of the absorbed nitrogen were used for growth. The growth secured, however, was equivalent to only 34.6 mg., so that the utilization for growth was  $34.6 \div 39.8 = 87.0$  percent. On a ration, containing approximately 10 per cent of milk proteins, the average daily intake of absorbed nitrogen was 129.7 mg., with an average biological value of 84.8. The average maintenance requirement in these experiments was 17.6 mg. per rat per day, and the growth secured was equivalent to 92.4 mg. Assuming again that the utilization of milk nitrogen for maintenance at this higher level is still 100, the utilization of nitrogen for growth would equal  $92.4 \div (129.7 - 17.6) = 82.4$ . This is lower than the estimated utilization of milk protein for growth at a 5 per cent level of intake (87.0), indicating that other factors are operating in the lowering of the biological value of milk proteins at higher levels of intake, than a mere difference in the proportion of the absorbed nitrogen used for maintenance.

The first explanation that might occur, is that the greater the rate of influx of amino-acids to the tissues from the digestive tract the less the economy with which they are handled. If this were

true, however, it would be expected that the utilization of dietary nitrogen in metabolism would be increased, at certain levels of intake at least, by increasing the number of feedings and thus assuring a more constant and less rapid influx of amino-acids to the tissues. However, Chanutin and Mendel (5) have recently shown that with dogs the utilization of casein, at a low and a high level of intake, is unaffected by the manner of feeding; *i.e.*, whether all the day's food is taken in a single feeding or in fractional feed-

TABLE XII

*Nitrogenous Metabolism of Rats as Affected by Single and Fractional Feeding.*

Nitrogen content of ration	Length of period	Feedings per day	Rat 1 Average weight 210 gm 7.50 gm food daily.			Rat 2 Average weight 250 gm 8.70 gm food daily			Rat 3 Average weight 60 gm 3.60 gm food daily.		
			Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen intake	Urinary nitrogen	Fecal nitrogen
per cent	days		mg	mg	mg	mg	mg	mg	mg	mg	mg.
2.992	5	1	209*	185	30	260	179	60	108	60	19
	5	3	209*	178	48	260	168	52	108	57	20
	5	1	209*	193	39	260	197	62	108	59	22
1.531	5	1	115	95	25	133	87	39	55	30	12
	5	3	115	88	28	133	88	29	55	30	11
	5	1	115	81	32	133	80	39	55	28	15
1.043	5	1	78	51	22	91	66	25	37	21	10
	5	3	78	50	23	91	51	34	37	20	13
	5	1	78	49	22	91	51	36	37	18	12
	5	6	78	51	26	91	45	35	37	19	11
	4	1	78	45	31	91	47	34	37	18	12

\* The food intake of Rat 1 was 7.0 gm. per day in these three periods.

ings. We have obtained similar data on six rats, agreeing with Chanutin and Mendel at low levels of intake, the percentage of nitrogen in our low protein rations being considerably less than that in the low protein rations of Chanutin and Mendel. With our high protein rations, however, containing a percentage of nitrogen much smaller than the high protein rations of Chanutin and Mendel, all six rats showed a higher utilization of nitrogen in

metabolism in the period of fractional feeding as indicated by the urinary excretion than in the preceding and following periods in which the day's food was consumed in one feeding. The data of this experiment are summarized in Tables XII and XIII.

In this experiment the protein mixture fed was the same qualitatively in all periods, being furnished by a mixture of corn and tankage in the ratio of 85 to 15. The first ration used, containing 2.992 per cent of nitrogen, contained only this mixture of feeds.

TABLE XIII

*Nitrogenous Metabolism of Rats as Affected by Single and Fractional Feeding.*

Nitrogen content of ration	Length of period	Feedings per day	Rat 3 Average weight 147 gm 6 00 gm food daily			Rat 4 Average weight 138 gm 6 00 gm food daily			Rat 5 Average weight 140 gm 6 00 gm food daily		
			Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen intake	Urinary nitrogen	Fecal nitrogen
per cent	days		mg	mg	mg	mg	mg	mg	mg	mg	mg.
2 992	4	1	179	110	32	179	101	33	179	104	28
	4	3	179	105	33	179	93	39	179	99	38
	4	1	179	113	25	179	111	37	179	123	35
1 531	4	1	92	55	23	92	49	26	92	49	22
	4	3	92	55	22	92	47	24	92	52	23
	4	1	92	50	23	92	50	27	92		
1 043	4	1	63	36	20	63	32	19	63	32	17
	4	3	63	32	20	63	34	18	63	32	22
	4	1	63	35	14	63	33	18	63	32	23
	4	6	63	34	17	63	39	20	63	32	19
	4	1	63	35	19	63	33	20	63	32	24

The rations with smaller percentages of nitrogen were obtained by mixing with the first ration varying amounts of a synthetic non-protein ration, containing adequate amounts of minerals and vitamins. In all periods, each rat received the same amount of feed, with the one exception noted in the table, this amount being just sufficient, as determined in a preliminary period of several weeks duration, to maintain the rat at constant weight. On the days in which only one feeding was given, the entire amount was

invariably consumed within 30 minutes, generally in a much shorter time. On the other days, the fractional feedings were distributed throughout the day as evenly as practicable, excepting for the period from 11 p.m. to 6 a.m. Following a reduction in the nitrogen content of the ration, collection of urine and feces was interrupted for at least 3 days.

Since our 5 and 10 per cent protein rations contained only 0.8 to 1.6 per cent of nitrogen, the data in Tables XII and XIII afford no grounds for suspecting that the rate of absorption of a given amount of protein affected in any way the economy of its utilization in metabolism. Another factor that may plausibly operate in reducing the biological value of a given protein or protein mixture as its concentration in the ration increases, is an inevitable wastage of amino-acids in the oxidative processes of the cells. It seems unreasonable to suppose that amino-acids of dietary origin are totally immune to oxidation, or are oxidized only in so far as they are not used in anabolism, even though enough non-protein material is being consumed to cover, or more than cover, the energy demands of the body. If they are not thus totally immune to oxidation, the extent of their oxidation will depend upon their concentration in the cellular fluids with respect to the concentration of non-nitrogenous nutrients, which would in turn be largely dependent on the percentage of protein in the ration. If this wastage of amino-acids by oxidation increases more rapidly than the protein intake, a greater percentage loss of absorbed nitrogen, due to inevitable oxidation, will result with rations containing higher percentages of protein. A greater proportional specific dynamic effect of protein at moderate than at low levels of intake would result in this situation. This explanation, therefore, ascribes the increase in the biological value of protein as the level of intake decreases to a true protein-sparing effect of carbohydrate.

It is interesting to note in this connection that the one food for which a constant biological value of its nitrogen was obtained, was the potato, containing from 40 to 60 per cent of its nitrogen in non-protein forms.

The opinion of Martin and Robison, expressed in their recent paper on the biological value of proteins (6), that the "validity of the method adopted by Thomas for the determination of the biological values of proteins depends in the first place on the uni-

formity of this value when varying amounts of the same protein are consumed," is not convincing. It can be defended only on the assumption that the biological value of a protein is fixed by its

TABLE XIV

*Biological Value of the Proteins of the Navy Bean at a 10 Per Cent Level.*

Rat No	Initial weight	Final weight	Food intake	Nitrogen intake	Fecal nitrogen	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine	Absorbed nitrogen retained in body	Biological value
	gm	gm	gm	mg	mg	mg	mg	mg	mg	mg	per cent
160	159	157	8 37	141 6	35 8	19 0	122 6	82 2	65 6	57 0	47
161	154	152	6 32	106 9	34 3	21 9	85 0	76 5	59 7	25 3	30
162	137	142	8 18	138 4	38 4	24 4	114 0	85 7	71 7	42 3	37
163	133	130	8 62	145 8	32 4	17 6	128 2	90 7	73.5	54 7	43
164	138	140	5 73	97 0	28 4	13 8	83 2	66 4	54 4	28 8	35
Average biological value . . . . .											38 4

TABLE XV

*Biological Value of the Proteins of Tankage at a 10 Per Cent Level.*

Rat No	Initial weight	Final weight	Food intake	Nitrogen intake	Fecal nitrogen.	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine	Absorbed nitrogen retained in body	Biological value.
	gm	gm	gm	mg	mg	mg	mg	mg	mg	mg	per cent
124	113	113	9 69	144 5	68 3	45 6	98 9	89 7	62 4	36 5	37
	113	107	7 41	110 5	51 6	34 5	76 0	77 0	50 4	25 6	34
125	88	80	6 91	103 0	50 1	34 4	68 6	66 4	47 0	21 6	31
	80	78	5 36	79 9	43 6	31 4	48 5	54 4	37 7	10 8	22
126	111	105	8 11	120 9	51 3	31 4	89 5	82 1	59 3	30 2	34
	105	99	6 60	98 4	41 3	25 9	72 5	70 3	50 1	22 4	31
Average biological value . . . . .											31 5

chemical constitution. The total neglect of many possible physiological factors that may modify the biological value of a protein, some of which have been mentioned above, renders this assumption untenable.



## 922 Biological Value of Proteins—Results

In Tables XIV and XV are given the metabolism data on the proteins of the navy bean (after steam-cooking) and the proteins of the packing house by-product known as tankage. These protein mixtures are the lowest in biological value of any that we have thus far tested.

### SUMMARY.

The biological values of the proteins (total nitrogen) of milk, corn, oats, rice, yeast, potatoes, navy beans, and the packing house by-product tankage, have been determined by a modified Thomas method described and defended in the preceding paper.

With the proteins of corn, milk, oats, and potatoes, this determination was made with rations containing approximately 5 and approximately 10 per cent of protein ( $N \times 6.25$ ) and, with the exception of potatoes, the biological values were smaller at the higher level. The probable factors responsible for this reduction in biological value are discussed on the basis of available experimental data.

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## THE SUPPLEMENTARY RELATIONS AMONG PROTEINS.

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The problem of assessing the biological value of proteins and protein mixtures is complicated, not only by the fact that it varies with the concentration of protein in the ration, but also by the fact that the biological value of a mixture of proteins is not necessarily the mean of the biological values of the individual proteins, each value being weighted according to the proportion in which the protein occurs in the mixture. We may consider each protein fed at a given low level as consisting of two fractions, one including the maximum proportion of amino-acids that can be used to replenish or enlarge the supply of nitrogenous substances in the tissues, the other including the remaining proportion of amino-acids destined to be deaminized, because it does not contain the complete assortment of amino-acids essential for synthesis into complexes needed by the tissues. If two proteins are fed together to an animal, those fractions of each which would otherwise be deaminized may together contain a complete assortment of amino-acids, permitting a part of the combined fractions to be used for synthetic purposes. In such a case, the biological value of the mixture would be greater than the weighted mean of the biological values of both.

The case may be illustrated in the simplest way by the nitrogen balance data when casein is fed as the sole protein in the ration, and when it is supplemented with cystine, which seems to be the amino-acid limiting the utilization of this protein in anabolism. Such data on a single rat are summarized in Table I.

The values in the last three columns are estimated as described fully in the first paper of this series. Evidently the small addition of cystine has rendered available for structural purposes a relatively large amount of casein nitrogen that otherwise would have been excreted.

## 924 Supplementary Relations among Proteins

A clear case of a supplementary relation existing between two protein mixtures is contained in Table II. These results were obtained on a group of five rats fed on a ration containing approximately 10 per cent of protein, three-fourths of which were corn

TABLE I  
*Effect of an Addition of Cystine on the Biological Value of Casein.\**

Initial weight	Final weight	Food intake	Cystine nitrogen consumed	Nitrogen intake	Food nitrogen in feces	Food nitrogen in urine	Biological value
<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>per cent</i>
118	124	9 09	0 00	80 1	6 3	18 6	75
128	127	6 96	1 44	62 7	7 7	4 4	92
126	126	6 80	1 02	61 0	8 8	7 2	86

\* The results in this and the following tables are expressed on the daily basis.

TABLE II  
*Supplementary Relation between Milk Proteins and Corn Proteins.*

Rat No	Initial weight	Final weight	Food intake	Nitrogen intake	Fecal nitrogen	Food nitrogen in feces	Absorbed nitrogen	Urnary nitrogen	Food nitrogen in urine	Absorbed nitrogen retained in body	Biological value
	<i>gm.</i>	<i>gm</i>	<i>gm</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>per cent</i>
65	135	147	7 79	118 3	23 2	6 8	111 5	52 2	29 6	81 9	73
	151	162	8 69	132 0	24 5	3 8	128 2	61 3	32 8	95 4	74
66	105	119	7 36	111 8	22 5	6 7	105 1	51 1	24 8	80 3	77
	126	129	6 96	105 7	24 8	9 8	95 9	50 7	16 7	79 2	82
67	126	139	7 86	119 4	30 1	12 6	106 8	51 7	28 9	77 9	73
	146	155	8 39	127 4	28 5	9 8	117 6	56 4	26 1	91 5	78
68	116	127	7 21	109 5	23 2	4 3	105 2	49 8	27 9	77 3	73
	139	147	8 54	129 7	30 2	7 8	121 9	56 3	27 3	94 6	78
69	111	123	7 00	106 3	19 8	5 1	101 2	50 9	24 7	76 5	76
	122	130	7 47	113 5	21 6	7 4	106 1	57 1	28 5	77 6	73

Average biological value . . . . . 75.7

protein and one-fourth, milk protein. The average biological value of the mixed proteins in ten 1 week periods was 75.7. The same rats were fed 10 per cent rations of corn and milk proteins separately, the data being given in Tables VIII and IX of the preceding paper. The average biological value for corn proteins was 61.3, and for milk proteins 84.7. The weighted mean of these

two values for a mixture of 3 parts of corn protein and 1 part of milk protein is 67.1. The difference between 67.1 and 75.7, the value actually obtained, represents the supplementary action of one protein mixture on the other.

A summary of the biological values obtained with each of these five rats on 10 per cent rations of corn proteins alone, milk proteins alone, and a mixture of the two is given in Table III.

TABLE III

*Biological Values of Corn Proteins Alone, Milk Proteins Alone, and a Mixture of the Two in the Proportion of 3 to 1, All Rations Containing Approximately 10 Per Cent of Protein.*

Protein	Rat No.					Average
	65	66	67	68	69	
Corn . .	58	63	62	63	64	62 0
	55	65	60	62	61	60 6
Milk . .	85	85	86	81	83	84 0
	84	87	87	83	86	85 4
Corn and milk (actual)	73	77	73	73	76	74 4
	74	82	78	78	73	77 0
Corn and milk (estimate)	63 5	69 5	67 4	67 4	68 0	67 2

The figures at the bottom of the table are weighted means of the average biological values for corn and milk proteins separately. In each case this estimate of the biological value of the mixture of corn and milk proteins is considerably less than the values actually obtained, indicating a distinct supplementary relation between the two.

We have also demonstrated a distinct supplementary relation between corn proteins and the proteins of tankage. In farm practice, good results are obtained in the growth and fattening of swine by feeding a mixture of corn and tankage.<sup>1</sup> The value of

<sup>1</sup> The latter feed contains packing house refuse, including meat scraps, condemned meat, bones, blood, and viscera, that has been submitted to prolonged treatment with steam under pressure for the removal of most of its grease. The product is then dried and finely ground. In regulating the composition of tankage, attention is paid only to total nitrogen, the content of which is varied by varying the proportion of blood and of the evaporated liquid ("stick") obtained in the drying of previous batches of tankage.

## 926 Supplementary Relations among Proteins

the latter feed in supplementing corn is commonly supposed to be twofold: first, in contributing an abundance of calcium and phosphorus; and, second, in contributing an abundance of protein, of which the best grades of tankage contain 50 to 60 per cent. However, no information is available as to whether the value of tankage as a protein supplement depends simply on the intrinsic value of its proteins or on their supplementing effect on the proteins of corn. In the preceding paper (Table XV), duplicate results on three rats were reported relative to the biological value of tankage

TABLE IV

*Supplementary Relation between Corn Proteins and the Proteins of Tankage.*

Rat No	Length of period	Initial weight	Final weight	Food intake	Nitrogen intake	Fecal nitrogen	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine	Absorbed nitrogen retained in body	Biological value
	days	gm	gm	gm	mg	mg	mg	mg	mg	mg	mg	per cent
1	15	209	203	7 50	114 8	28 3	10 0	104 8	88 0	59 4	45 4	43
2	15	251	248	8 70	133 2	35 7	14 0	119 2	85 0	47 3	71 9	60
6	15	57	55	3 60	55 1	12 7	7 2	47 9	29 3	20 0	27 9	58
3	12	145	152	6 00	91 9	22 4	11 1	80 8	53 3	31 1	49 7	62
4	12	137	144	6 00	91 9	25 9	14 6	77 3	48 5	31 1	46 2	60
5	8	144	138	6 00	91 9	22 7	12 0	79 9	50 7	28 7	51 2	64
124	7	114	120	8.61	134 7	18 3	0 0	134 7	65 5	37 2	97 5	73
	7	120		7 81	122 1	15 6	0 0	122 1	67 0	39 0	83 1	68
125	7	80	86	5 80	90 7	15 6	2 4	88 3	54 9	38 9	49 4	56
	7	86		4 57	71 5	12 8	2 4	69 1	42 1	27 6	41 5	60
126	7	105	110	7 96	124 5	17 3	0 0	124 5	57 6	37 7	86 8	70
	7	110		7 93	124 0	15 5	0 0	124 0	61 1	42 1	81 9	66

proteins themselves fed at a 10 per cent level. This value seems to be exceptionally low, averaging 31.5. However, when tankage and corn are mixed in such proportions that the protein of the mixture is derived equally from the two feeds, the biological value of the combined proteins is fully equal to that of corn proteins and possibly is even higher. In Table IV is given a series of determinations of the biological value of the proteins of such a mixture, fed at a 10 per cent level. With one exception, these values cluster closely about an average of 60, which is the average biological

value of corn proteins. In the second half of the table are given duplicate determinations on the same three rats used in the tankage experiments just referred to. While the individual determinations do not agree particularly well among themselves, they do indicate that the mixed proteins have at least as high a biological value as the proteins of corn alone. This, of course, would not be the case unless a distinct supplementary relation existed between the proteins of corn and the proteins of tankage. It would seem,

TABLE V  
*Supplementary Relation between Gelatin and Oat Proteins.*

Rat No.	Initial weight	Final weight	Food intake	Nitrogen intake	Fecal nitrogen	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Food nitrogen in urine	Absorbed nitrogen retained in body	Biological value
	gm	gm	gm	mg	mg	mg	mg	mg	mg	mg	per cent
60	211	210	10 11	164 3	33 3	12 2	152 1	123 1	83 8	68 3	45
	210	206	9 49	154 2	30 1	10 9	143 3	121 5	83 4	59 9	42
61	229	230	11 96	194 4	36 9	12 6	181 8	143 5	104 4	77 4	43
	230	231	11 27	183 1	27 3	5 4	177 7	139 5	98 8	78 9	44
62	228	230	11 31	183 8	34 6*	13 1	170 7	143 1	103 0	67 7	40
	230	227	10 29	167 2	25 3	7 1	160 1	134 7	93 7	66 4	41
63	222	225	10 69	173 7	30 4	9 9	163 8	126 9	93 0	70 8	43
	225	223	9 64	156 6	29 4*	11 9	144 7	119 3	85 0	59 7	41
64	209	210	10 93	177 6	32 6	6 4	171 2	126 0	87 2	84 0	49
	210	202	9 24	150 1	27 6*	5 9	144 2	128 6	90 1	54 1	38
Average biological value											42 6

\* The nitrogen determinations on these feces were lost. The values given in the table were obtained by assuming an average digestibility of the dietary nitrogen.

therefore, that the value of tankage as a protein supplement for corn depends on a distinct increase in the protein content of the ration, but not upon any appreciable increase in the biological value of the protein.

We have not been able to establish any certain supplementary relation between gelatin and oat proteins. Such a relation has been indicated by experiments reported by McCollum, Simmonds, and Pitz (1), and more certainly, by the quantitative results of Osborne and Mendel (2). In Table X of the preceding paper is

given a summary of the results of duplicate tests on a group of five rats on a 10 per cent oat protein ration. Immediately after this test was completed, the rats were put upon a ration containing 10 per cent of protein, approximately three-fourths of which were derived from oats and one-fourth from gelatin. After the usual period of preliminary feeding, two successive balance periods were run, the results of which are summarized in Table V. While oat proteins alone had an average biological value of 64.9, the mixture of oat proteins and gelatin in the ratio of 3 to 1, had an average biological value of only 42.6. The individual values in this experiment were remarkably concordant. It is evident, therefore, that in our experience the mixture of oat proteins and gelatin was distinctly inferior to oat proteins alone. It is, however, difficult to determine whether a slight supplementary relation may not exist between oat proteins and gelatin, since the biological value of the latter protein would not be comparable in any way to that of complete proteins. Our attempts to determine the biological value of gelatin in covering the maintenance requirement of rats have given widely discordant values, ranging from 0 to 44, an experience similar to that of Robison (3).

While many instances of supplementary relations between animal and vegetable proteins have been indicated or demonstrated by feeding experiments or metabolism studies, few instances of an appreciable supplementary effect of the proteins of one vegetable product upon those of another have been reported, in so far as the author is aware. Nevens has reported from this laboratory the results of combining in all possible ways the proteins of corn, cottonseed meal, and alfalfa (4); for only one combination, *i.e.* that of cottonseed meal and alfalfa, was any such effect indicated, and this effect was slight. In another report (5) we have shown that no supplementary relations exist between the proteins of coconut meal and rice bran, nor between the proteins of corn and coconut meal, though between the proteins of corn and soy beans there may be a slight supplementary effect.

#### SUMMARY.

The existence or non-existence of a supplementary relation between the proteins of different feeds, and the extent of such a relation if it exists, may be readily determined from the results

of metabolism studies on rats so designed as to permit of the calculation of the biological values of the proteins of each food separately and of different combinations of them.

In this manner, a considerable supplementary relation has been shown to exist between the proteins of corn and of milk, and between the proteins of corn and of tankage. No supplementary relation could be demonstrated between gelatin and the proteins of the oat kernel.

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## CLINICAL CALORIMETRY.

### XXXIV. KETOSIS AND THE RESPIRATORY EXCHANGE IN DIABETES.

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#### INTRODUCTION.

This paper is concerned with a study of the following questions: (1) In what proportions must foodstuffs be oxidized by the diabetic in order to prevent ketosis? (2) To what extent is ketosis affected by changes in the diet? (3) What is the effect of fasting on ketosis and on the carbohydrate metabolism? These questions are important in the treatment of diabetes with or without insulin. In order to get the full benefit of the internal secretion of the pancreas, whether derived from his own gland or injected

subcutaneously, the diabetic must use as much fat as is necessary within safe limits. To this end an understanding of the ketogenic action of fat is essential.

### *Literature.*

An excellent review of the extensive literature on antiketogenesis may be found in Shaffer's second paper (4, b). Only a few investigations may be mentioned here. In 1910 Woodyatt (1) stated the problems involved, and advanced a theory as to its mechanism. He credited the alcohol or  $-OH$  radical, and Ringer and Frankel (2) credited the aldehyde or  $-CHO$  radical with the power of antiketogenesis. Ladd and Palmer (3), working with diabetic patients, calculated from the diets the ratio of available carbohydrate to fat at which the elimination of ketones first showed a marked increase, and found it to be approximately 1 to 4. Papers by Shaffer (4) and Woodyatt (5) in 1921 presented a detailed theory of antiketogenesis, the underlying principle being that the ketones are formed in the course of the normal metabolism and are completely oxidized, provided that they come into contact with a sufficient quantity of glucose which is itself in the process of oxidation. In the absence of this "oxidizing" glucose they accumulate in the tissues. Both Shaffer and Woodyatt<sup>1</sup> published formulas by which to estimate the ketogenic balance. The formula of Woodyatt is expressed in terms of fatty acid and glucose, and reads as follows:

$$\begin{array}{rcl} \text{Fatty acid} & & 0.46 \times \text{protein} + 0.9 \times \text{fat} \\ \text{Glucose} & - & \text{Carbohydrate} + 0.58 \text{ protein} + 0.1 \times \text{fat} \end{array}$$

All the constituents of the formula are expressed in grams. The ratio obtained by this formula may be converted into the molecular form by dividing it by the appropriate molecular weights. For fatty acid this may be taken as the average weight of oleic and palmitic acids, or 270 (5, b). The molecular weight of glucose is 180. 270 divided by 180 is 1.5. Therefore, in order to obtain the molecular ratio divide the Woodyatt ratio by 1.5. Consequently, when the Woodyatt ratio is 1.5, a molecular ratio of 1 to 1 is indicated.

In his third article (4) Shaffer has constructed a table by which the ketogenic balance, expressed as a molecular ratio, can be read off direct from the respiratory quotient, thus making his analysis readily applicable to the data of respiratory observations. He has since (4) modified the formula by increasing the ketogenic value ascribed to protein. By means of this

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<sup>1</sup>These two formulas are based on identical assumptions, which have been very clearly described by Shaffer (4, b). Provided that the calculations are based on the foodstuffs metabolized, the choice of one formula or the other is largely a matter of personal convenience.

change, together with the assumption that 1 molecule of glucose is the equivalent of 2 of keto-acid he has succeeded in making the calculations agree with the quantity of ketones actually excreted in cases of severe ketosis. The method to be described below is not adapted to such cases, and the remaining references will be confined to the question of the ketogenic balance at the *threshold* of ketosis. Woodyatt (5), Shaffer (4, b), and McCann, Hannon, Perlzweig, and Tompkins (6) found the threshold of ketosis near a ratio of 1 molecule of keto-acid to 1 of glucose, whereas Wilder and Winter (7) and Ladd and Palmer (3) found it at a ratio of more than 1 molecule of keto-acid to 1 of glucose. Wilder, Boothby, and Beeler (8) found that with their patient the critical ratio was not the same at different levels of the metabolism.

The principle that ketosis depends on the excess of ketogenic over antiketogenic substances receives confirmation from the work of Joslin (9), who found that the lower the respiratory quotient, and therefore the smaller the proportion of glucose oxidized, the greater the ketosis. In only 8 of the 66 experiments with patients with severe ketosis was the quotient greater than 0.76. This corresponds accurately to the level found by Shaffer at the border-line of ketogenesis.

The production of ketones by normal fasting individuals has also been explained by both of these authors on the basis of the ketogenic balance.

### *Method.*

The method was the same as that used in a previous investigation in this laboratory (10). The respiration calorimeter was used in conjunction with the urinary nitrogen to determine the quantities of protein, fat, and carbohydrate oxidized. In order to make the period of the calorimeter observation a fair sample of the 24 hours the diet was given in small portions every 2 hours. The observations from Nov. 22 on were begun on the average 36 minutes after the last meal, and usually lasted 3 hours. On two other occasions one 2 hour portion of food was given before the patient entered the calorimeter, and another after he had been in it 2 hours, an average of 5 hours being taken. The results were essentially the same by either method.

Observations were controlled by means of alcohol checks, which ~~are recorded~~ in Table I. The respiratory quotients for 3 hour periods ranged from 0.66 to 0.68, as compared to the theoretical quotient for alcohol of 0.667. The error in the oxygen ranged from 0 to -3.6 per cent. If this maximum error of 3.6 per cent be applied to the calculation of 800 non-protein calories per day found in a typical patient of our series, the difference would be

TABLE I  
*Alcohol Check*

Date	Hour	Alcohol burned	Heat.			Oxygen			Carbon dioxide			Water.			R. Q. (theory 0.667).
			Theory	Found	Error	Theory	Found	Error	Theory	Found	Error	Theory	Found	Error	
1922 Nov. 17		gm	cal	cal	per cent	gm	gm	per cent	gm	gm	per cent	gm	gm	per cent	
	1	7.92	56.1	57.2	+2.0	16.5	16.3	-1.2	15.1	15.1	±0.0	10.1	10.8	+6.9	0.675
	2	8.00	56.6	56.3	-0.5	16.7	16.3	-2.4	15.3	14.9	-2.6	10.1	10.7	+5.9	0.664
	3	7.78	55.1	55.0	-0.2							9.9	10.4	+5.1	
	Average		55.9	56.2	+0.5	16.6	16.3	-1.8	15.2	15.0	-1.3	10.0	10.6	+6.0	0.669
1923 Jan. 8	1	6.41	45.4	45.8	+0.9	13.4	13.2	-1.5	12.2	12.1	-0.8	8.1	8.8	+8.6	0.667
	2	6.47	45.8	45.8	±0.0	13.5	13.1	-3.0	12.4	11.9	-4.0	8.2	8.4	+2.4	0.656
	3	6.37	45.1	45.3	+0.4	13.3	13.4	+0.8	12.2	12.0	-1.6	8.1	8.5	+4.9	0.652
	Average		45.4	45.6	+0.4	13.4	13.2	-1.5	12.3	12.0	-2.4	8.1	8.6	+6.2	0.658
Jan. 10	1	6.33	44.8	44.4	-0.9	13.2	13.5	+2.3	12.1	11.9	-1.7	8.0	8.7	+8.8	0.644
	2	6.52	46.1	45.4	-1.6	13.6	13.2	-2.9	12.5	12.2	-2.4	8.3	8.6	+3.6	0.672
	3	6.15	43.5	44.7	+2.7	12.8	13.0	+1.6	11.8	11.9	+0.8	7.8	8.4	+7.7	0.663
	Average		44.8	44.8	±0.0	13.2	13.2	±0.0	12.1	12.0	-0.8	8.0	8.6	+7.5	0.660
Jan. 12	1	7.92	56.1	55.5	-1.1	16.5	16.3	-1.2	15.1	15.0	-0.7	10.1	10.6	+5.0	0.669
	2	8.00	56.6	57.0	+0.7	16.7	16.4	-1.8	15.3	15.2	-0.7	10.1	10.7	+5.9	0.671
	3	7.92	56.1	55.5	-1.1	16.5	15.9	-3.6	15.1	14.7	-2.6	10.1	10.5	+4.0	0.675
	Average		56.3	56.0	-0.5	16.6	16.2	-2.4	15.2	15.0	-1.3	10.1	10.6	+5.0	0.672

Feb. 5	1	7 18	50 9	51 1	+0 4	15 0	14 1	-6 4	13 7	13 6	-0 7	9 1	9 4	+3 3	0 699
	2	7 11	50 3	47 4	-5 8	14 8	14 3	-3 4	13 6	13 3	-2 2	9 0	9 3	+3 3	0 676
	3	7 18	50 9	50 7	-0 4	15 0	14 8	-1 3	13 7	13 5	-1 4	9 1	9 3	+2 2	0 661
	Average		50 7	49 7	-1 9	14 9	14 4	-3 4	13 7	13 5	-1 5	9 1	9 3	+2 2	0 679
Feb. 6	1	7 26	51 4	50 9	-1 0	15 1	14 8	-2 0	13 9	13 8	-0 7	9 2	9 4	+2 2	0 679
	2	7 04	49 8	50 2	+0 8	14 7	14 5	-1 4	13 5	13 4	-0 7	8 9	9 4	+5 6	0 672
	3	6 89	48 8	50 1	+2 7	14 4	14 3	-0 7	13 2	13 0	-1 5	8 7	9 1	+4 6	0 660
	Average		50 0	50 4	+0 8	14 7	14 5	-1 3	13 5	13 4	-0 7	8 9	9 3	+4 6	0 670
Mar. 27	1	4 01	28 4	30 5	+7 2	8 4	8 7	+3 6	7 7	7 8	+1 3	5 7	6 6	+15 8	0 652
	2	4 21	29 8	30 2	+1 3	8 8	8 5	-3 4	8 1	7 7	-4 9	6 0	6 0	±0 0	0 651
	3	4 08	28 9	30 8	+6 6	8 5	8 2	-3 5	7 8	7 8	±0 0	5 8	6 4	+10 4	0 690
	Average		29 0	30 5	+5 2	8 6	8 5	-1 2	7 9	7 8	-1 2	5 8	6 3	+8 6	0 664
Apr. 26	1	7 92	56 1	54 8	-2 3	16 5	16 5	±0 0	15 1	15 0	-0 6	10 1	10 8	+6 9	0 661
	2	7 85	55 6	54 8	-1 4	16 4	15 8	-3 7	15 0	14 9	-0 6	10 0	10 5	+5 0	0 686
	3	8 07	57 2	56 0	-2 1	16 8	15 8	-6 0	15 4	14 7	-4 5	10 2	10 4	+2 0	0 677
	Average.		56 3	55 2	-2 0	16 6	16 0	-3 6	15 2	14 9	-2 0	10 1	10 6	+5 0	0 674

29 calories. The maximum error from the oxygen should therefore not exceed 29 non-protein calories a day.

To eliminate as far as possible the chance that the metabolism of the diabetic might not be the same by night as by day, two observations were made with George H., one beginning about noon on Apr. 19, and the other at midnight 36 hours later. The time which had elapsed since the last meal was the same in the two instances. The total metabolism, as may be seen from Table II, decreased, from 1,261 to 1,168 calories per 24 hours, but the quotient changed only from 0.74 to 0.75. Moreover, the relation between the fatty acid-glucose ratio and the excretion of the acetone bodies were maintained at night during sleep, as well as in the daytime.

The calculations were based on the carbon dioxide eliminated by the patient, the oxygen absorbed, and the nitrogen of the urine during a calorimeter observation of from 2 to 5 hours. Using the nitrogen excretion and the table of Zuntz and Schumburg the quantities of protein, fat, and carbohydrate oxidized during the calorimeter period were computed and expressed as calories per 24 hours. They were then converted into grams and used to compute the fatty acid-glucose ratio by means of the formula of Woodyatt (5, a). The total quantity of glucose oxidized during the metabolism of these food materials was also calculated, using Woodyatt's formula for "G." This method differs from that of Woodyatt in one essential; namely, that the calculations were based on the metabolism and not on the diet. The latter appears in the following paper only for the purpose of comparison.

### *Case Histories.*

*Case 1.*—Moderately severe diabetes Frank B., age 34 years, condiment maker, born in Austria, was admitted to the metabolism ward on Apr. 17, 1922. His symptoms began 6 or 8 months before admission. Physical examination showed him to be undernourished, flushed, with acetone odor of the breath. His blood sugar ranged from 422 to 130 mg. per 100 cc. The maximum excretion of the acetone bodies was 2.27 mg. On Apr. 21 a boil about 4 cm. in diameter was incised, and 4 cc. of pus were evacuated. He developed a tolerance for 100 gm. of carbohydrate, exclusive of glucose derived from protein or fat.

*Case 2.*—Moderate diabetes. Frank C., age 28 years, accountant, was admitted to the metabolism ward for the second time on Nov. 10, 1922. His diabetic symptoms began in Jan., 1921. The physical examination was

negative except for absent knee-jerks and evidence of slight enlargement of the liver. His blood sugar while in the ward ranged from 214 to 131 gm. per 100 cc. The maximum excretion of the acetone bodies was 3.30 gm. on a high fat diet, and the lowest carbon dioxide-combining power of the blood was 59 vol. per cent. He developed a tolerance for 40 gm. of carbohydrate, exclusive of protein and fat.

*Case 3.*<sup>1</sup>—Severe diabetes. Harold J., age 9 years, born in the United States, was admitted to the metabolism ward for the first time on Jan. 5, 1922. The severity of his case at that time was shown by the dextrose-nitrogen ratio which exceeded 3 on several occasions. He was readmitted on Nov. 14, 1922, having been treated with insulin since Aug. of the same year. On withholding the insulin his excretion of acetone rose to 1.96 gm. with a diet containing 60 gm. each of protein and fat, and 6 gm. of carbohydrate. The carbon dioxide-combining power of the blood at this time was 42 vol. per cent. The blood sugar was 242 gm. per 100 cc. on the 5th day of withdrawal of insulin.

*Case 4.*—Mild diabetes. Chris Q., age 35 years, fireman, born in the United States, was admitted to the metabolism ward on Dec. 23, 1922. The symptoms of diabetes dated back 8 months. The physical examination was negative except for evidence of some loss of weight. His blood sugar on Dec. 14 and 15 ranged from 227 to 126 mg. per 100 cc. The maximum excretion of the acetone bodies was 2.01 gm. on a high fat diet. The lowest carbon dioxide-combining power of the blood was 54 vol. per cent. He developed a tolerance for 50 gm. of carbohydrate while in the ward.

*Case 5.*—Moderately severe diabetes. Morris G., age 24½ years, born in Roumania, was admitted to the metabolism ward on Jan. 9, 1923. He had been treated for diabetes for 2 years. The physical examination was negative except for considerable loss of flesh. His greatest excretion of the acetone bodies was 2.24 gm. on a balanced diet, and the lowest carbon dioxide-combining power of the blood was 40 vol. per cent. He was unable to tolerate without insulin a diet greater than the lowest Newburgh diet of 14 gm. of carbohydrate and 935 calories.

*Case 6.*—Moderate diabetes, with improvement. Ray H., age 15 years, schoolboy, born in the United States, was admitted to the metabolism ward on Jan. 25, 1923. He had symptoms of diabetes for a month previous, beginning shortly after an attack of sore throat. The physical examination was essentially negative except for pallor, emaciation, and apathy. The greatest excretion of the acetone bodies was 2.74 gm. on a balanced diet. The lowest carbon dioxide-combining power of the blood was 50 vol. per cent. With a rapid increase in tolerance he was able to take 50 gm. of carbohydrate without glycosuria.

*Case 7.*—Mild diabetes. David L., age 27 years, hospital messenger, was observed in the metabolism ward at intervals during the months of

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<sup>1</sup> Opportunity to study this patient was given through the kindness of Dr. Frederick M. Allen.



TABLE II  
Calorimeter Data in Terms of Averages per Hour

Date Weight. Surface area	Time of observation	CO <sub>2</sub>	O <sub>2</sub>	R Q	N <sub>2</sub> in urine	Indirect calories	Pulse	Work added *	Remarks Diet in gm
Frank B									
Apr. 19, 1922. 41 9 kg 1.43 sq. m.	11 47 a m to 1 47 p m	20 5	19 9	0 75	1 045	64 3	68	18	Breakfast at 10 a m Protein 32, fat 13, carbohydrate 25.
Apr. 20, 1922. 43 2 kg. 1 44 sq. m.	11 43 a m to 1 43 p m	16 2	15 7	0 75	0 498	51 0	61	20	Restless. Fasting 24 hrs.
Apr. 21, 1922. 43 7 kg. 1 45 sq. m.	11 47 a m to 1 47 p m.	15 4	14 6	0 76	0 632	47 7	61	11	Fast continued to 48 hrs. Quiet.
Apr. 25, 1922. 45 4 kg. 1.48 sq. m.	12 07 p.m. to 2 07 p m.	16 9	14 8	0 83	0 578	49 0	64	5	Food at 2 hr intervals, last meal at 10 a.m. Quiet. Protein 98, fat 71, carbohydrate 22, per 24 hrs.
Apr. 26, 1922. 42.5 kg. 1 44 sq. m.	11 58 a.m. to 1 58 p m.	14 8	13 4	0 80	0 345	44 3	58	10	Basal after 3 days diet of protein 82, fat 59, carbohydrate 18, per 24 hrs. Fairly quiet.

Frank C.									
Apr. 28, 1922. 42 g. kg. 1.45 sq. m.	11 58 a. m. to 1 58 p. m.	16 1	14 0	0 84	0 712	46 1	60	5	Breakfast at 6 a. m. Protein 33, fat 13, carbohydrate 25. Quiet.
Nov. 21, 1922. 46 g. kg. 1.53 sq. m.	12 20 p. m. to 2 20 p. m.	14 1	12 8	0 80	0 170	42 8	61	4	Fasting 24 hrs.
Nov. 23, 1922. 46 g. kg. 1.53 sq. m.	11 22 a. m. to 2 22 p. m.	14 2	13 0	0 81	0 168	43 3	62	8	Fasting 24 hrs.
Nov. 28, 1922. 47.2 kg. 1.53 sq. m.	10 47 a. m. to 1 47 p. m.	15 0	13 6	0 80	0 169	45 5	73	3	Protein 26, fat 69, carbohydrate 67, per 24 hrs. ‡ at 10 a. m. Very quiet.
Dec. 5, 1922. 46.5 kg. 1.52 sq. m.	11 35 a. m. to 1 35 p. m.	14 5	13 1	0 80	0 280	43 5	62	6	Basal. Quiet.
Dec. 7, 1922. 49.3 kg. 1.56 sq. m.	10 26 a. m. to 2 26 p. m.	16 5	14 2	0 85	0 480	47 4	63	4	Food at 2 hr. intervals; last meal at 10 a. m. Protein 44, fat 63, carbohydrate 69, per 24 hrs.

\* The work adder is an instrument by which the activity of the patient is measured. A very quiet patient raises the work adder less than 5 cm. and a very restless patient, more than 25 cm.

TABLE II—Continued.

Date. Weight. Surface area.	Time of observation	Q̄	Q̄	Q̄	R Q	N <sub>2</sub> in urine.	Indirect calories	Pulse.	Work added.*	Remarks Diet in gm.
Harold J.†										
Dec. 1, 1922. 15.69 kg.	12.26 p.m. to 2.26 p.m.	gm 8 21	gm. 7 67	gm 0 78	0 78	265	25 1	61	17	Basal. Diet of previous day; protein 54, fat 60, carbohydrate 6. Restless.
Dec. 4, 1922. 15.6 kg.	12.06 p.m. to 2.06 p.m.	7 73	7 30	0 77	0 77	272	24 6	60	7	Quiet. Basal. Diet of previous day; protein 60, fat 60, carbohydrate 6.
Chris Q.										
Dec. 14, 1922. 57.7 kg. 1.68 sq. m.	11 19 a.m. to 1.19 p.m.	18.3	17 6	0 76	0 76	446	57 5	58	8	Fairly quiet; 24 hr. fast.
Dec. 15, 1922. 58.4 kg. 1.68 sq. m.	10 32 a.m. to 1 32 p.m.	19 8	18 8	0 77	0 77	650	61 4	55	10	Food at 2 hr. intervals; last meal at 10 a.m. Protein 69, fat 100, carbohydrate 36, per 24 hrs. Restless during last hr.

## Morris G.

Jan. 16, 1923. 46 8 kg. 1.50 sq. m.	12 47 p.m. to 2 47 p.m.	13.7	12 9	0 77 0	398	42 4	56	10	Food at 2 hr. intervals; last meal at 12 n. Protein 40, fat 60, carbohydrate 20, per 24 hrs. Quiet.
Jan. 18, 1923. 46 4 kg. 1.49 sq. m.	11 58 a.m. to 1 58 p.m.	13 0	12 0	0 78 0	322	39 6	58	6	24 hr. fast. Quiet.
Jan. 19, 1923. 47.2 kg. 1.51 sq. m.	10 36 a.m. to 1 36 p.m.	13 7	13 1	0 76 0	481	42 8	57	5	Protein 50, fat 60, carbohydrate 45, per 24 hrs. Food at 2 hr. intervals; last meal at 10 a.m. Quiet.
Jan. 22, 1923. 45.6 kg. 1.48 sq. m.	12 38 p.m. to 3 38 p.m.	13 1	12 4	0 77 0	250	40 7	57	8	Moved frequently during the last hr. Food at 2 hr intervals; last meal at 12 n. Protein 10, fat 90, carbohydrate 14, per 24 hrs.
Jan. 25, 1923. 48 8 kg. 1 50 sq. m	10 34 a.m. to 3 34 p.m.	13 6	13 2	0 75 0	209	43 3	61	11	Food at 2 hr. intervals; last meals at 10 a.m. and 12 40 p m Protein 10, fat 90, carbohydrate 14, per 24 hrs.

## David L.

Feb. 2, 1923. 54 6 kg. 1.61 sq. m.	10 40 a.m. to 12 40 p.m.	17 7	16 4	0 79 0	485	53 8	58	8	Basal Quiet.
Feb. 12, 1923. 54 7 kg. 1.61 sq. m.	11 48 a.m. to 1 48 p.m.	17 6	15 4	0 83 0	102	52 0	53	8	Basal. Quiet.

† Height = 122.3 cm.

TABLE II—*Concluded.*

Date Weight. Surface area.	Time of observation	CO <sub>2</sub>	O <sub>2</sub>	R Q	N <sub>2</sub> in urine	Indirect calories	Pulse	Work added *	Remarks Diet in gm.
Ray H.									
Jan. 26, 1923. 32 7 kg. 1 24 sq. m.	10 49 a.m. to 1 49 p.m.	gm 13 4 13 1	gm 11 4	0 75 0 340	gm 42 7	65	5		Basal. Quiet. Diet of previous day; protein 26, fat 40, carbohydrate 14.
Jan. 29, 1923. 33 6 kg. 1.25 sq. m.	12 18 p.m. to 3 18 p.m.	12 0	11 4	0 77 0 168	37 5	60	4		30 hr fast, after 3 days of diet; protein 40, fat 60, carbohydrate 20, per 24 hrs. Very quiet.
Jan. 30, 1923. 33 1 kg. 1.25 sq m.	11.23 a.m. to 2 23 p.m.	11 6	10 9	0 78 0 171	35 9	51	5		Quiet Fast continued to 54 hrs.
Feb. 9, 1923. 33 7 kg. 1 26 sq. m.	10 41 a.m. to 3 41 p.m.	13 5	12 7	0 77 0 201	42 0	68	6		Food at 2 hr intervals, last meals at 10 a.m. and 12 45 p.m Protein 27, fat 126, carbohydrate 42, per 24 hrs Restless during 5th hr.
Mar. 1, 1923 32.3 kg. 1.23 sq. m.	11 53 a.m. to 1 53 p.m.	12 6	10 8	0 85 0 218	36 3	72	6		Basal after a diet of protein 70, fat 200, carbohy- drate 70, per 24 hrs. Quiet.

## Jervis B.

Mar. 14, 1923. 52 7 kg. 1 64 sq. m.	12 18 p. m. to 2 18 p. m.	18 8	18 0	0 76	0 313	59 2	60	10	Basal. Fairly quiet.
Mar. 15, 1923. 52 8 kg. 1 64 sq. m.	11 39 a. m. to 2 39 p. m.	17 7	18 1	0 71	0 338	58 8	60	17	Fasting continued to 2 days.
Mar. 16, 1923. 52 8 kg. 1 64 sq. m.	11 24 a. m. to 2 24 p. m.	17 1	17 3	0 72	0 229	56 7	60	13	Fasting continued to 3 days. Quiet.
Mar. 19, 1923. 54 5 kg. 1 66 sq. m.	11 53 a. m. to 2 53 p. m.	14 9	15 3	0 71	0 125	49 9	54	16	Fasting continued to 6 days. Quiet 2 hrs. Restless last hr.

## George H

Apr. 19, 1923. 54 2 kg. 1 67 sq. m.	1 12 p. m. to 4 12 p. m.	16 3	16 0	0 74	0 202	52 6	61	13	Basal. Quiet. Diet of previous day; protein 40, fat 90, carbohydrate 14.
Apr. 21, 1923. 54 4 kg. 1 67 sq. m.	12 40 a. m. to 4 40 a. m.	15 3	14 8	0 75	0 195	48 7	55	8	Basal at night. Asleep all but 20 min. Diet of preceding 24 hrs.; protein 30, fat 61, carbohy- drate 11

TABLE III

*Excretion of the Acetone Bodies in Relation to the Calories Ingested, the Calories Oxidized, and the Fatty Acid-Glucose Ratio Calculated from the Calorimeter Data.*

Date.	Diet per 24 hr.				Metabolized per 24 hr.				Metabolized fatty acid-glucose ratio from Columns 6, 7, and 8	Urine acetone bodies in 24 hr specimen.
	Protein.	Fat.	Carbohydrate	Total.	Protein	Fat.	Carbohydrate.	Total.		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)

Frank B.										
1922	cal.	cal.	cal	cal	cal	cal	cal	cal.		gm.
Apr. 18										2 27
" 19	387	372	118*	1,068	665	871	0	1,536	1 5	1.21
" 20	0	0	0	0	317	824	84	1,225	1 6	0.54
" 21	0	0	0	0	402	651	88	1,141	1 3	0.32
" 25	401	660	89	1,150	368	422	383	1,172	0 6	0.35
" 26	0	0	0	0	220	565	278	1,063	0 8	0.20
" 28	310	372	261*	1,092	453	311	342	1,106	0 5	0.10

Frank C.										
Nov. 21	0	0	0	0	108	622	297	1,026	0 8	0 49
" 23	0	0	0	0	107	662	269	1,038	0 9	0 26
" 28	107	642	275*	1,023	108	660	325	1,092	0 7	0 27
Dec. 5	0	0	0	0	178	584	284	1,046	0 8	0 61
" 7	178	587	260*	1,025	305	386	446	1,137	0 5	0 22

Harold J.										
Dec. 1	0	0	0	0	169	344	90	603	1 1	0 76
" 4	0	0	0	0	173	348	70	590	1 2	1.01

Chris Q.										
Dec. 14	0	0	0	0	284	951	146	1,381	1 4	2.07
" 15	285	949	148	1,382	414	891	171	1,474	1 2	0.81

Morris G.										
1923										
Jan. 16	162	558	20*	740	253	618	146	1,017	1.1	0.81
" 18	0	0	0	0	205	563	183	951	1.0	0.84
" 19	207	558	81*	845	306	635	86	1,028	1.4	0.72
" 22	39	837	59	935	159	656	162	978	1.2	0 81
" 25	39	837	59	935	133	799	108	1,040	1.7	0.86

TABLE III—*Concluded.*

Date.	Diet per 24 hr.				Metabolised per 24 hr.				Metabolised fatty acid-glucose ratio from Columns 6, 7, and 8.	Urine acetone bodies in 24 hr. specimen.
	Protein.	Fat.	Carbohydrate.	Total.	Protein.	Fat.	Carbohydrate.	Total.		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)

## Ray H.

1923	cal.	cal	cal.	cal.	cal	cal.	cal.	cal		gm.
Jan. 26	0	0	0	0	216	742	66	1,024	1 8	2.74
" 29	0	0	0	0	107	634	160	901	1 2	0 66
" 30	0	0	0	0	109	584	170	862	1.1	0.34
Feb. 9	108	1,171	157*	1,436	128	701	180	1,009	1.2	0.62
Mar. 1	0	0	0	0	139	343	389	871	0 4	0.13

## David L.

Feb. 2	0	0	0	0	309	738	245	1,291	1.0	0 0†
" 12	0	0	0	0	65	681	503	1,248	0 5	0.0†

## Jervis B.

Mar. 14†	0	0	0	0	132	1,030	259	1,420	1 2	7 96
" 15†	0	0	0	0	149	1,248	14	1,411	3.6	12.13
" 16†	0	0	0	0	104	1,212	43	1,359	3 4	9.14
" 19	0	0	0	0	80	1,119	0	1,198	5 1	2.24

## George H.

Apr. 19	0	0	0	0	128	1,029	104	1,261	2 0	1.24
" 20	0	0	0	0	124	902	142	1,168	1 6	0 80

\* After deducting the calories lost in the urine in the form of glucose.

† Daily average for the week including the calorimeter observation.

‡ The glucose of the urine was assumed to come from protein. The quantity of glucose appears in Table V.

Feb. and Mar., 1923. Throughout the observations he received an accurately measured diet from the diet kitchen of the Presbyterian Hospital. He was very carefully studied by Ladd and Palmer (3).<sup>\*</sup> The onset of symptoms late in the summer of 1921 was acute, with a considerable degree of acidosis, but he improved rapidly under treatment. During the winter of 1922-1923 he continued to do well, and remained fit for his work as a

<sup>\*</sup> For the records of this case see Ladd and Palmer (3), Case XV, p. 5.



messenger. At the time of the calorimeter observations his greatest blood sugar was 171 mg. per 100 cc., and the lowest carbon dioxide-combining power of the blood was 63 vol per cent. With a diet of 50 gm. of protein, 243 gm. of fat, and 80 gm. of carbohydrate in 24 hours he had no appreciable quantity either of glucose or of acetone in the urine.

*Case 8.*—Severe diabetes. Jervis B, age 27 years, laborer, born in Greece, was admitted to another ward of the Bellevue Hospital on Mar. 9, 1923. Symptoms of diabetes began a year previous. Physical examination on admission showed him to be extremely drowsy, with long and regular respirations, and acetone odor of the breath. The ferric chloride test of the urine was positive. The blood sugar on two occasions was over 400 mg. per 100 cc. The carbon dioxide-combining power of the blood was 30 vol. per cent on Mar. 12. By this time the patient was much improved and he was transferred to the metabolism ward. On Mar. 15 the carbon dioxide of the blood had increased to 48 per cent. The acetone bodies of the blood, expressed in mg. of acetone per 100 cc., were 54, 57, 61, and 41 on Mar. 14, 15, 16, and 19, respectively. Other laboratory data are shown in the tables. His clinical condition continued to improve while he was in the ward.

*Case 9.*—Moderate diabetes. George H, age 28 years, clerk, born in the United States, was admitted in the metabolism ward on Apr. 17, 1923. The onset of diabetic symptoms was in Dec. 1922. Physical examination revealed nothing abnormal except evidence of loss of flesh. The maximum excretion of the acetone bodies on a high fat diet was 2.40 gm. The lowest carbon dioxide-combining power of the blood was 52 vol per cent. His blood sugar was 136, and again 143 mg per 100 cc. He developed a tolerance for at least 30 gm. of carbohydrate.

### *Results of Experiments.*

The details of the calorimeter observations appear in Table II.

*I. The Threshold of Ketosis in Relation to the Proportions of the Foodstuffs Oxidized.*—Table III shows the excretion of the acetone bodies in relation to the oxidation of foodstuffs as determined by the calorimeter. The food ingested is also shown, the carbohydrate being corrected by subtracting the calories lost in the urine in the form of glucose, allowing 3.7 calories per gram. Columns 2 to 5 inclusive relate to the diet, and Columns 6 to 10 to the calorimeter data. The length of the observations varied from 2 to 5 hours, and appears in Table II. The acetone was determined in 24 hour specimens of urine, although the results were similar if only the urine which was voided during the calorimeter period was taken, and the figures recalculated for 24 hours (Table VI). Ketosis is arbitrarily defined in this paper as the excretion in the urine in 24 hours of 1 gm. or more of acetone bodies ex-

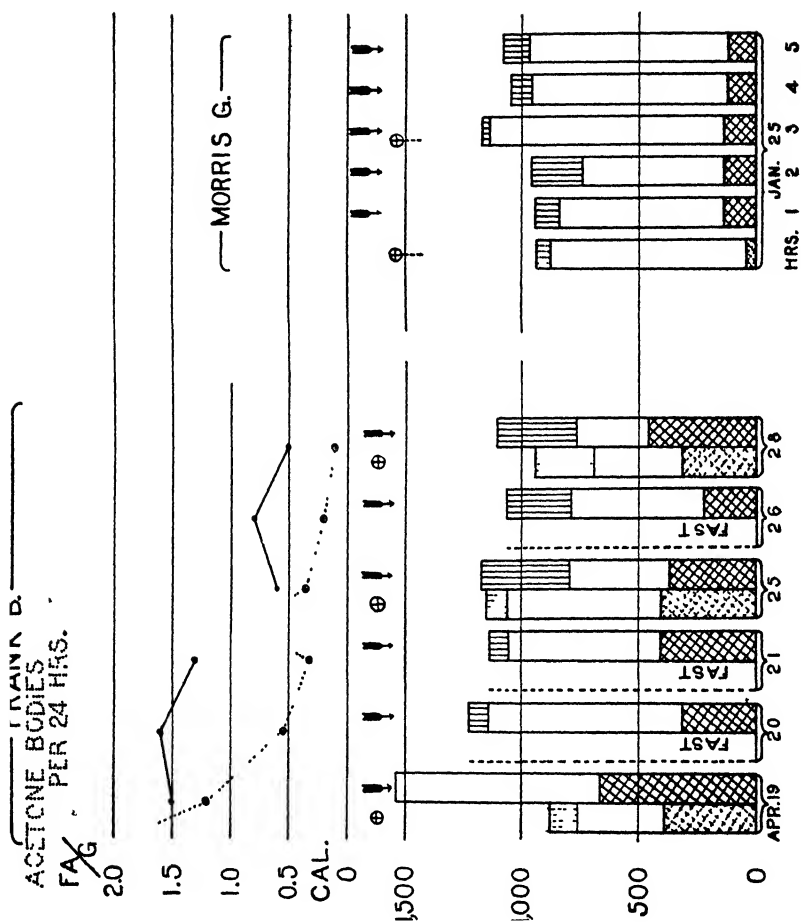
pressed as acetone. The lowest fatty acid-glucose ratio at which ketosis occurred was 1.2 with Harold J. on Dec. 4, and the highest at which it failed to occur was 1.7 with Morris G. on Jan. 25. With few exceptions ratios above 1.5 were attended with ketosis, and ratios below it were not. The critical ratio at which it first occurred was, therefore, between 1.2 and 1.7, in other words not far from the value of 1.5, at which in theory 1 molecule of keto-acid is present for each molecule of glucose oxidized.

In graphic form, as presented in Fig. 1, the data show a close parallelism between the ratio and the excretion of the acetone bodies. Frank B. shows a falling excretion at a ratio of 1.5. Ray H. passes the threshold of ketosis when the ratio is passing 1.5. Low ratios are in general associated with low excretions, and *vice versa*.

These results agree well with unpublished data of the previous year in which the critical ratio at which ketosis first occurred was found to lie between 0.9 and 1.7. The criteria for ketosis were somewhat different in the earlier work, their presence being judged from a rising excretion, and their absence from the reverse.

*II. The Threshold of Ketosis in Relation to the Diet.*—Diets in which the high content of fat would be expected to produce ketosis often failed to do so. The reason for the failure is that they were not oxidized in the same proportions in which they were ingested. These statements are based on a previous investigation (10) and also on the data of Table III and Fig. 1. Included in these are three observations with the Newburgh type of diet, namely the last two on Morris G. and the last one, Feb. 9, on Ray H. The graph shows that in these instances, as well as most of the others, more fat was ingested than was oxidized. Similarly, on closer analysis by means of the formula of Woodyatt the proportion of fatty acid to glucose was usually found to be higher if calculated from the diet than if calculated from the calorimeter data, as may be seen from Table IV. Not infrequently it was twice as high. Since in our experiments the excretion of the acetone bodies was found to be related not to the diet, but only to the metabolic ratio,<sup>4</sup> it is evident that the diet is not to be implicitly trusted as a basis for calculating the ketogenic balance.

<sup>4</sup> We have used the term metabolic ratio to denote the fatty acid-glucose ratio calculated from the calorimeter data.



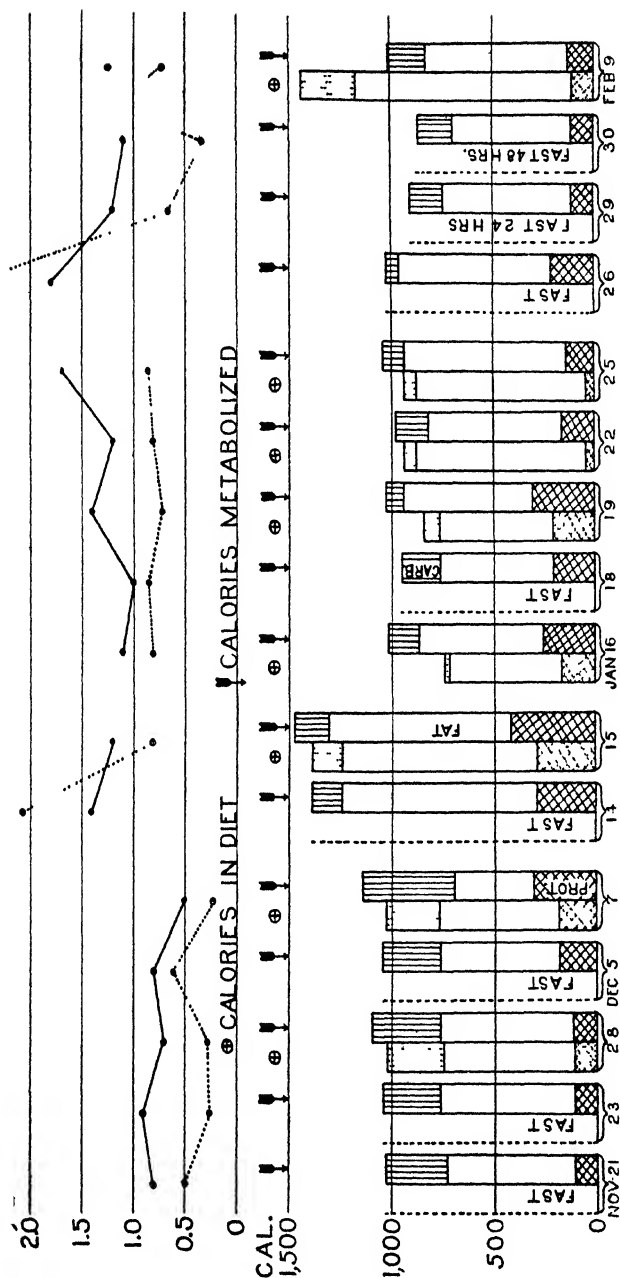


FIG. 1. Showing the relation between the excretion of the acetone bodies and the fatty acid-glucose ratio, the diet, and the foodstuffs oxidized.

The total height of the columns represents the total calories of diet or metabolism. The cross-hatched portion at the bottom represents protein, the middle portion, which is blank, represents fat, and the upper portion, vertically shaded, represents carbohydrate. The diet is marked by circles, and further, by broken shading. When no food was given the fact is indicated by the word "fast." The calories metabolized are designated by arrows. The curves drawn in solid lines represent the fatty acid-glucose ratio calculated from the calorimeter data. The broken lines represent the

In this connection two points need emphasis: first, that the diets were for the most part about 1,000 calories a day, never exceeded 1,500, and seldom exceeded the basal metabolism; second, that by the time the patients received the diets they had already been treated by undernutrition in some form. With untreated patients or heavier diets the discrepancy might have been of different degree.

*III. The Influence of Fasting on Ketosis.*—It has been a puzzle why fasting in diabetes is attended with a fall in ketosis, whereas

TABLE IV  
*The Fatty Acid-Glucose Ratio.*

Name	Date	Diet *	Metabolized
	1922		
Frank B.	Apr 25	1 3	0 6
Frank C.	Nov. 28	0 9	0 7
	Dec 7	0 8	0 5
Chris Q.	" 15	1 4	1 2
	1923		
Morris G.	Jan. 16	2 2	1 1
	" 19	1 5	1 4
	" 22	3 0	1 2
	" 25	2 7	1 7
Ray H.	Feb. 9	1 9	1 2

\* Allowing for the calories lost in the urine in the form of glucose.

in normal individuals it is attended with a rise. This paradox may be explained by the fatty acid-glucose ratio as calculated from the calorimeter data. As shown in Table III and Fig. 1, Frank B. in the course of 2 consecutive days of fasting showed a fall both in the metabolic ratio and in the excretion of acetone bodies. During the 2nd day both were reduced as compared to the level of the last day of diet, Apr. 19. There was a still further drop at the time of a subsequent fast. With Ray H. the ratio and the excretion fell simultaneously while he was fasting. Chris Q., on the other hand, had a decrease both in the ratio and in the elimination of ketones when he resumed his diet. Although his metabolism was probably influenced by the high fat diet which he received beforehand, nevertheless the observation shows that fasting in diabetes is not necessarily attended

with less ketosis than is a diet. It may be inferred that fasting diabetic patients, like the others, pass the threshold of ketosis in either direction, in accordance with the proportions of the foodstuffs which they oxidize.

*IV. The Influence of Fasting on the Metabolism of Glucose.*—The data of Table V and Fig. 2 show that with the progress of fasting the oxidation of the glucose derived from all sources, in other words, the "G" of Woodyatt's formula, underwent no significant increase. The gain in the oxidation of carbohydrate as such, which was observed with the first two patients, was offset by the loss in the oxidation of glucose derived from protein and the glycerol of the fat. These relations are shown also in Fig. 1. With all three of the patients there was a reduction of blood sugar and of urinary glucose. That this fall did not carry with it an increase in the oxidative power of the patient may seem paradoxical, but a simple explanation is available and will be discussed below.

In contrast with their metabolism while fasting, the patients manifested a definite increase in their ability to oxidize glucose after they had resumed food. Ray H. on Mar. 1, oxidized a total of 118 gm., although the absence of glucose from the urine on this date implied that this was not the utmost of which he was capable. This amount compares favorably with the maximum of 63 gm. which he oxidized during the fast. In like manner, Frank B. showed an improvement during the restricted diet which followed the fast, as shown by the oxidation of 151 gm. of glucose on Apr. 28 in contrast to a maximum of 85 gm. while fasting.

A point which has received little attention in the study of the carbohydrate metabolism in the diabetic is that there are really two questions involved; first, how much glucose *does* he oxidize, and second, how much *can* he oxidize? It is with the second question that we are concerned here, for it is evident that even a normal individual can be starved until he oxidizes no carbohydrate whatever. When a diabetic excretes glucose it may be inferred that he is oxidizing all of this substance which he is able. This was the case while fasting with all three of the patients shown in Table V, except for Ray H. on the last day of the fast.

TABLE V  
*Effect of Fasting on the Metabolism of Glucose.*

(1) Date	(2) Carbohydrate oxidized, ex- trived from the glucose de- rived from the oxidation of protein and fat.	(3) Fatty acid calculated from the calorimeter data by the formula of Woodgast.	(4) Glucose oxidized as a result of the metabolism of pro- tein, fat, and carbohy- drate, calculated from the calorimeter data by the method of Woodgast.	(5) Blood sugar	(6) Glucose in urine.	(7) Remarks Diet in gm.
Frank B.						
1923	gm. per 24 hrs.	gm. per 24 hrs.	gm. per 24 hrs.	mg. per 100 cc.	gm. in 24 hrs.	
Apr. 18	0	159	103	422	105	On diet.
" 19					52	Breakfast at 10 a.m.; protein 32, fat 13, carbohydrate 25.
" 20	20	115	74	245	10	Fasting 24 hrs.
" 21	21	108	85	173	Trace.	Fast continued to 48 hrs.
" 25	93	83	150	219	"	Food at 2 hr. intervals; protein 98, fat 71, carbohydrate 22, per 24 hrs.
" 26	68	79	105	130	"	Basal after 3 days diet of protein 82, fat 59, carbohydrate 18, per 24 hrs.
" 28	84	81	151	234	40	Breakfast at 6 a.m.; protein 33, fat 13, carbohydrate 25.

## Ray H.

1923						
Jan. 25				441	150+	Protein 26, fat 40, carbohydrate 14, per 24 hrs.
" 26	16	96	55		48	Basal. Diet of previous day; protein 26, fat 40, carbohydrate 14.
" 29	39	73	61	205	Trace.	30 hr. fast after 3 days of protein 40, fat 60, carbohydrate 20, per 24 hrs.
" 30	41	69	63	207	0	Fast continued to 54 hrs.
Feb. 9	44	82	69	91	4	Food at 2 hr. intervals; protein 27, fat 126, carbohydrate 42, per 24 hrs. for 9 days.
Mar 1	95	49	118	116	0	Basal, after protein 70, fat 200, carbohydrate 70, per 24 hrs.

## Jervis B.\*

Mar. 14	63	122	90	326	18	Basal
" 15	3	145	28	263	18	Continuation of fast to 2 days.
" 16	10	134	33	157	11	Fasting continued to 3 days.
" 19	0	118	18	229	Trace.	Continuation of fast to 6 days.

\* The calculation used by Lusk for the total diabetic was employed for this patient, since his case was very severe. As pointed out by Shaffer (4,c) this method is applicable to any subject. It gives the quantity of glucose metabolized during the oxidation of protein as well as carbohydrate. To this we have added the glucose derived from the glycerol of the fat.



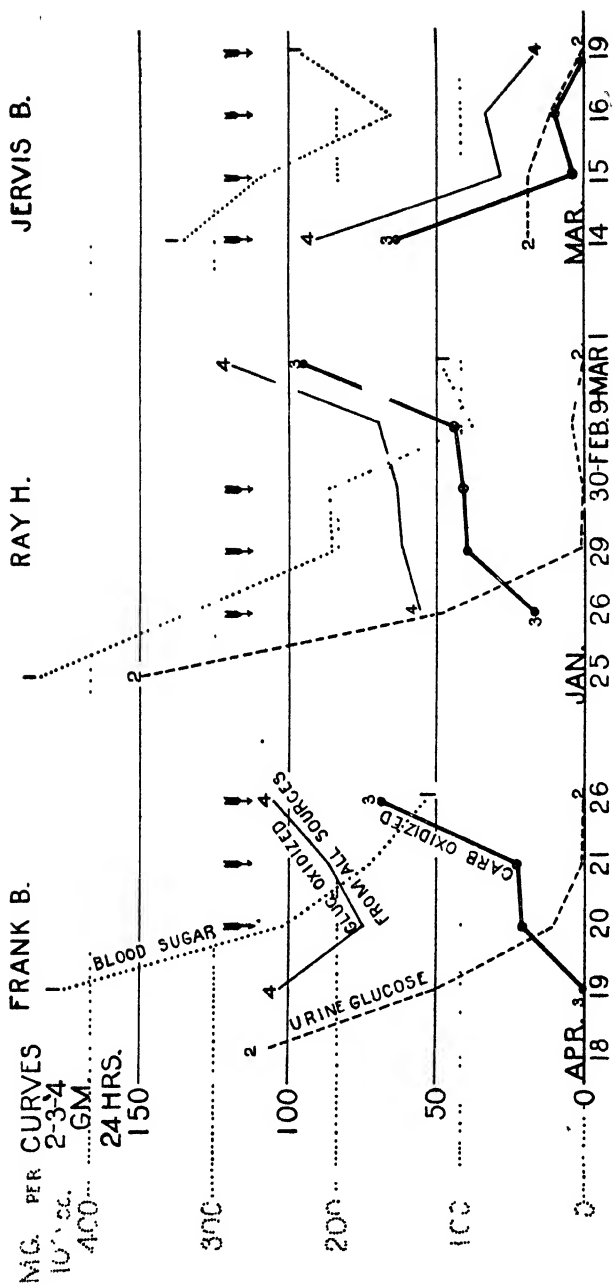


FIG. 2. The effect of fasting on the metabolism of glucose.

Curve 1 represents blood sugar, Curve 2, glucose in the urine in grams in 24 hours; Curve 3, carbohydrate, not including the glucose from protein and fat, calculated from the calorimeter data and expressed in grams per 24 hours; Curve 4, oxidation of glucose derived from all sources, including protein and the glycerol of the fat, calculated from the calorimeter data by means of the formula of Woodyatt, and expressed in grams per 24 hours.

The arrows point to observations on patients without food.

TABLE VI.

*The Ketogenic Balance Calculated from the Calorimeter Data by Different Methods; Its Relation to the Excretion of Acetone Bodies.*

Date (1)	Ratio of gm of fatty acid to gm of glu- cose (Woodyatt)	Ratio of molecules of keto-acid to mole- cules of glucose (Column 2 - 1.5)	Ratio of molecules of keto-acid to mole- cules of glucose, from R Q (Shaf- fer)	Respiratory quotient	Total acetone bodies in 24 hr specimen, as acetone	Total acetone bodies per 24 hrs in calor- imeter specimen, as acetone.
Frank B						
1922					gm	gm.
Apr. 19	1 5	1 0	1 2	0 75	1 21	
" 20	1 6	1 1	1 2	0 75	0 54	
" 21	1 3	0 9	1 0	0 76	0 32	
" 25	0 6	0 4	0 3	0 83	0 35	
" 26	0 8	0 5	0 5	0 80	0 20	
" 28	0 5	0 3	0 3	0 84	0 10	
Frank C						
Nov. 21	0 8	0 5	0 5	0 80	0 49	
" 23	0 9	0 6	0 4	0 81	0 26	
" 28	0 7	0 5	0 5	0 80	0 27	
Dec 1	0 8	0 5	0 5	0 80	0 61	0 14
" 7	0 5	0 3	0 3	0 85	0 22	0 15
Harold J.						
Dec 1	1 1	0 7	0 7	0 78	0 76	0 20
" 4	1 2	0 8	0 8	0 77	1 01	0 53
Chris Q						
Dec. 14	1 4	0 9	1 0	0 76	2 07	2 40
" 15	1 2	0 8	0 8	0 77	0 81	1 07
Morris G.						
1923						
Jan. 16	1 1	0 7	0 8	0 77	0 81	0 81
" 18	1 0	0 7	0 7	0 78	0 84	0 66
" 19	1 4	0 9	1 0	0 76	0 72	0 96
" 22	1 2	0 8	0 8	0 77	0 81	0 81
" 25	1 7	1 1	1 2	0 75	0 86	0 42

TABLE VI—*Concluded.*

Data. (1)	Ratio of gm. of fatty acid to gm. of glu- cose (Woodyatt)	Ratio of molecules of keto-acid to mole- cules of glucose (Column 2 + 15)	Ratio of molecules of keto-acid to mole- cules of glucose, from R. Q. (Shal- fer).	Respiratory quotient	Total acetone bodies in 24 hr specimen, as acetone.	Total acetone bodies per 24 hrs. in calor- imeter specimen, as acetone.
Ray H.						
1925					gm.	gm.
Jan. 26	1 8	1.2	1.2	0 75	2 74	1 28
" 29	1 2	0 8	0 8	0 77	0 66	0 22
" 30	1 1	0 7	0 7	0 78	0 34	0 20
Feb. 9	1 2	0 8	0 8	0 77	0 62	0 45
Mar. 1	0 4	0 3	0 3	0 85	0 13	0 13
David L.						
Feb. 2	1 0	0 7	0 6	0 79	0 0*	0 21
" 12	0 5	0 3	0 3	0 83	0 0*	0 05
Jervis B.						
Mar. 14	1 2	0 8	1 0	0 76	7.96	9 51
" 15	3 6	2 4	6 2	0 71	12 13	12 5
" 16	3 4	2 3	3 2	0 72	9 14	8 10
" 19	5 1	3 4	6 2	0 71	2 24	2 57
George H.						
Apr. 19	2 0	1 3	1 6	0 74	1 24	0.96
" 20	1 6	1 1	1 2	0 75	0 80	0.46

\* Daily average for the week including the calorimeter observation.

The fall in the fatty acid-glucose ratio which accompanied fasting in the case of Frank B. and Ray H. was due not to an increase in the oxidation of glucose, but rather to a decrease in the fatty acid. It will be seen from Table V, Columns 4 and 3, that the glucose remained practically constant, whereas the fatty acid dropped sharply. The reduction of ketosis was brought about in these cases by a reduction in the total metabolism without evidence of an increase in the power of the patient to oxidize glucose.

TABLE VII.

*Effect of the Ingestion of Food on the Respiratory Quotient in Diabetes.*

Date.	Time elapsed between meal and beginning of observation.	Hour.	R Q.
Frank C.			
1922	min.		
Nov. 21	Fasting.	I	0 80
		II	0.80
Nov. 28	47	I	0.83
		II	0.83
		III	0.75
Dec. 5	Fasting	I	0 78
		II	0.82
Dec. 7	26	I	0 84
		II	0 84
		III	0 89
		IV	0 83
Chris Q.			
Dec. 14	Fasting	I	0.76
		II	0 76
Dec. 15	32	I	0 73
		II	0 78
		III	0.79
Morris G.			
1923			
Jan. 16	47	I	0 76
		II	0 79
Jan. 18	Fasting.	I	0.77
		II	0 80
Jan. 19	36	I	0 71
		II	0 78
		III	0 79
Jan. 22	38	I	0.73
		II	0.77
		III	0.81

TABLE VII.—*Concluded.*

Date.	Time elapsed between meal and beginning of observation.	Hour	R.Q
Morris G.—Concluded.			
1923	min.		
Jan. 25*	34	I	0 75
		II	0 79
		III	0 73
	54	IV	0 74
		V	0 75
Ray H.			
Jan. 30	Fasting	I	0 82
	"	II	0 76
	"	III	0.76
Feb. 9†	41	I	0 77
		II	0 80
		III	0 80
	56	IV	0 73
		V	0 77
Mar. 8‡	40	I	0 86
		II	0 83
		III	0 88
David L.			
Feb. 27§	Basal.	I	0 82
		II	0 76
	45	III	0 79
		IV	0 78

\* The subject took another meal 6 min after the beginning of the 3rd hour.

† The subject took another meal 4 min. after the beginning of the 3rd hour.

‡ Non-caloric "meal."

§ The subject took his first meal 15 min after the beginning of the 2nd hour.

V. *Comparison of Shaffer's and Woodyatt's Methods of Calculating the Ketogenic Balance.*—Shaffer (4, c) has published a table by which the ketogenic antiketogenic ratio may be read off directly from the respiratory quotient. Since his work and that of

Woodyatt (5, *a*) are based on identical principles, both methods should yield the same results if applied to the data of a respiratory observation, provided, of course, that both ratios are expressed in the molecular form. Dividing the Woodyatt fatty acid-glucose ratio by 1.5 we have obtained molecular ratios which correspond very closely to those obtained by means of Shaffer's table, as is apparent from inspection of Columns 3 and 4 in Table VI. The one method is based in part on the nitrogen of the urine, the other is not. This means that in the determination of the ketogenic balance by means of the calorimeter the metabolism of protein needs to be taken into account only in so far as it affects the total respiratory quotient. It follows that irregularities in the excretion of nitrogen could have had no influence on the final results.

*VI. Changes in the Respiratory Quotient Following the Ingestion of Food.*—Table VII demonstrates that with many patients the ingestion of food causes a marked depression of the respiratory quotient in the 1st hour of the observation, followed by a progressive rise in the succeeding periods. This change had no apparent relation with the composition of the diets, which may be found in Table II under "Remarks." The one thing which they had in common was an adequate quantity of fat and at least minimal amounts of protein and carbohydrate. The fall of the quotient was observed in four out of five patients. The constancy of the findings when the experiment was repeated, as well as the smoothness of the change, precludes the idea that it was accidental. Its effect upon the interpretation of the quotient in terms of the protein, fat, and carbohydrate oxidized in the successive hours is shown in Fig. 1 by Morris G. on Jan. 25. He received food before he entered the calorimeter and at the beginning of the 3rd hour inside it. Each time the food caused a sharp drop, real or apparent, in the quantity of carbohydrate oxidized.

#### DISCUSSION.

*Technique.*—The depression in the respiratory quotient often observed with diabetic patients following the ingestion of food has been taken into account in the foregoing work. It was for this reason that the observations were begun as soon as possible

after the last meal, and continued for 3 hours, and that on two occasions a second meal was given while the patient was in the calorimeter, and the observation continued for 5 hours. It may be pointed out, moreover, that enough experiments are available with patients under basal and fasting conditions to establish, independently of the diet, the level of the fatty acid-glucose ratio at which ketosis first occurs.

As in the previous paper (10) the results are strictly applicable only to patients at rest. If during exertion there is a change in the proportions of foodstuffs oxidized, without a corresponding change in ketosis, the results would be modified. Data bearing on this point are to be published from this laboratory later.

*Factors Affecting the Respiratory Quotient.*—The respiratory quotient is ordinarily assumed to measure accurately the oxidation of foodstuffs within the body. This may not be the case in severe ketosis. Except in the case of Jervis B., to be discussed later, the maximum excretion of acetone bodies on the days of the calorimeter observations was less than 3 gm., an amount quite insufficient to affect the respiratory quotient.

Other factors disturbing the respiratory quotient are conceivable. Thus, the conversion of carbohydrate into fat in the normal individual is known to be associated with a rise of quotient without necessarily any change in the proportion of carbohydrate oxidized. Joslin (9) has suggested this explanation for the high quotients observed by him in certain types of diabetes. Indeed, it is difficult to explain on any other basis the quotients of 1 or over which he observed following the ingestion of levulose. It does not follow, however, that the same explanation applies to patients under more normal dietary conditions. Particularly difficult is its application to the postabsorptive state, during which very much less carbohydrate is available for conversion into fat. With these patients another explanation for the high quotient is possible. They have an extremely low weight and metabolism compared to what would be expected of their age and height. The high quotient may be the result of the low metabolism, for it is evident that the oxidation of a given quantity of carbohydrate bears a greater proportion to the total than it would with a normal metabolism, and the respiratory quotient is correspondingly increased. For instance, Ben J.,

data concerning whom appear in a previous publication (10), had a maximum fasting quotient of 0.87. His weight was 34 per cent below the normal standard, and his metabolism 54 per cent below the level that would be expected of his age and height. He was little more than skin and bones, and at autopsy almost no fat could be found. His low metabolism and the absence of body fat are sufficient explanation why the oxidation of even a moderate amount of carbohydrate sufficed to produce a high quotient.

Krogh and Lindhard (11) have postulated a conversion of fat into carbohydrate to explain the diminished muscular efficiency which they observed with normal subjects who were oxidizing a large proportion of fat. This conversion would, however, be without effect on the respiratory quotient, provided that the material concerned were oxidized after its conversion. The net effect on the respiratory quotient would be the same at the end of complete oxidation whatever the path by which it proceeds.

The presence or absence of factors disturbing the respiratory quotient can be proved only by means of an independent method for measuring the oxidation of carbohydrate, a method as yet lacking. Oxidation of glucose is roughly indicated by lack of ketosis. High quotients indicating abundant oxidation of glucose, combined with ketosis indicating deficient oxidation, would point to a defect in the respiratory quotient as a measure of combustion. The analyses of Woodyatt and Shaffer on the basis of the respiratory data give opportunity to apply this same principle more accurately. If the fatty acid-glucose ratio is found in several patients consistently parallel with the excretion of the acetone bodies, and widely divergent in others, the divergence might point to a defect in the respiratory quotient as a measure of combustion. In none of our observations, except with the cases of very severe ketosis, to be discussed below, was such a deviation noted. Our evidence indicates that the respiratory quotient in periods of from 2 to 5 hours is a true measure of oxidative processes within the body.

The above analysis assumes that glucose requires oxidation in order to prevent ketosis. It might conceivably do so, even without oxidation, during its conversion into fat. Against this possibility is the evidence that *in vitro* it requires preliminary



treatment with alkali before it can promote the oxidation of acetoacetic acid (4, a). Moreover, in diabetes the glucose which is excreted unoxidized does not serve in its passage through the body to prevent ketosis. As judged by present knowledge an antiketogenic action of glucose during its conversion into fat is possible, but improbable.

*Discussion of Results.*—The ill effects of the unrestricted consumption of fat by diabetic patients in the early days led to exaggerated fear of its use. A great service has been performed by Shaffer, by Woodyatt, and by others, in taking the mystery out of the ketogenic action of fat, and defining the limits within which it can be used with safety. This paper describes the application of their methods to additional data obtained in the study of the respiratory exchange in diabetes. It was demonstrated with the calorimeter that under a wide variety of dietary conditions diabetic patients do or do not have ketosis in accordance with the proportions in which they oxidize foodstuffs. As analyzed by the formula of Woodyatt, the proportion at which ketosis occurred corresponded to a fatty acid-glucose ratio of 1.5, which in theory indicates the oxidation of 1 molecule of keto-acid for every molecule of glucose. The reason why diets with a calculated ratio much higher than this failed to produce ketosis was that the foodstuffs ingested were not oxidized in the same proportions as given. As compared to the diet the oxidation of fat, which is the main source of fatty acid, was decreased, and the oxidation of carbohydrate, which is the main source of glucose, was increased. It is not surprising, therefore, that the ratio as calculated from the diet was too high, often twice too high. The extent of the ketosis we have found dependent on the food oxidized, and not on the diet. This may be an explanation why Newburgh and Marsh (12) observed not an increase, but a diminution of ketosis on giving to patients their high fat diets. Safety in the use of this regime depends on the ability of the patient to contribute carbohydrate withdrawn from his own tissues, and to store fat. Since these factors cannot be estimated without the determination of the respiratory quotient, it is evident that close observation of the patient is required.

Caution in interpreting our results as favorable to the Newburgh regime is also demanded for the reason that we did not

use a diet exceeding 1,500 calories in 24 hours, nor did we study its effect on patients not previously treated. Clinical experience makes it seem to us improbable that had we done so similar results would have been obtained.

In this paper no attempt has been made to account for the actual number of grams or ketones excreted. Attention has been focussed on the ketogenic balance at the threshold of ketosis. The results agree closely with those obtained by Shaffer (4, b), Woodyatt (5), and McCann, Hannon, Perlzweig, and Tompkins (6); namely, that the ratio at the threshold of ketosis corresponds to the presence during oxidation of 1 molecule of keto-acid to 1 of glucose. Why, then, did Ladd and Palmer (3), Wilder, Boothby, and Beeler (8), and Wilder and Winter (7) find under like circumstances a ratio of more than 1 molecule of keto-acid to 1 of glucose? With some of the patients reported the disagreement may be explained by the choice of a different criterion for the threshold of ketosis. It is not surprising that relatively more ketogenic substances should be required to produce a high grade of ketonuria, than is required for a minimal one. Yet this explanation does not account for the entire difference. In an earlier paper (10) there has been pointed out the wide divergence which may exist between the diet and the foodstuffs metabolized, owing to the faculty of the organism for storage of food, and this may have affected the results obtained by Wilder and Winter. With the work of Ladd and Palmer, however, this criticism loses much of its force. At least one of their patients was kept on the same diet for periods of 2 to 4 weeks, during which he remained in equilibrium both as to nitrogen and body weight. Under these conditions the foodstuffs metabolized must have corresponded accurately to the diet, unless it be assumed that there was a replacement of glycogen by fat or *vice versa* during the entire period. A more probable reason than this for the difference in results is the factor of muscular exertion. Our patients were at absolute rest in bed. Ladd and Palmer's patient was employed as a messenger at the Presbyterian Hospital during the entire period of their observations. His daily heat production may be judged by the fact that he maintained a constant weight for over a year on a diet of 2,700 calories a day. Even allowing for the specific dynamic action of meals, nearly half of

his caloric output must be ascribed to muscular exertion, a factor which may conceivably exert a marked influence on the ketogenic balance.<sup>5</sup>

Two curves obtained with fasting diabetic patients showed a decrease with the progress of the fast both in the fatty acid-glucose ratio and the excretion of the acetone bodies. This correspondence has also been found during the past 2 years in observations which have included numerous cases of fasting. It was usually, but not necessarily, the case that ketosis diminished with fasting. Occasionally it did so with resumption of the food. This occurred with Chris Q. The evidence is that diabetic patients, fasting or otherwise, pass the threshold of ketosis in either direction in accordance with the proportions of the foodstuffs which they oxidize.

With very severe diabetes the above statements need qualification. Two such have been observed in this laboratory during the course of a prolonged fast; namely, Jervis B. (Table III) and Gerald S. (13). Except for one observation with each of these patients ketosis and a high ratio were both present at the same time, but they were not parallel. The fall of ketosis which was noted with the progress of the fast was not, however, associated with a corresponding change in the ratio. In other words, the calorimeter data failed to account for the improvement.

This failure may be only an apparent one, due to the difficulty in interpreting the respiratory quotient in presence of severe ketosis. The quotient is influenced on the one hand by acidosis, and on the other by the incomplete oxidation of protein and fat. Although these two factors work in opposite directions the net effect cannot be estimated with accuracy. This has been discussed by Lusk (14) and by Allen and Du Bois (13). In view of these difficulties, no inference is justified from the cases of Jervis B. and Gerald S. except that the output of ketones was very large, and the oxidation of carbohydrate slight or absent.

Turning to the effect of fasting on the metabolism of glucose no evidence was found during the fast of an increase in the ability

<sup>5</sup> Another explanation for the divergence of opinion on this subject has been given by Shaffer (4, c page 433), according to whom ketosis may result from a local surplus of ketogenic molecules, even in the absence of a surplus in the body as a whole.

of the patient to oxidize glucose provided that that protein and the glycerol of the fat were taken into account as sources of this substance. Yet the blood sugar fell rapidly even to the point at which glycosuria ceased. The explanation for this apparent contradiction is very simple. The glucose which was oxidized could come only from the body of the patient, and sooner or later its source must become depleted, just as the fuel in a lamp is used up as the flame burns. In other words, blood loses in sugar content simply because it is starved. According to this explanation the well known efficiency of fasting in rendering patients "sugar-free" consists in depletion of the endogenous sources of carbohydrate.

Since the oxidation of glucose did not change materially with the progress of fasting, the decrease in the fatty acid-glucose ratio was due chiefly to a fall in the fatty acid factor. In other words the metabolism of ketogenic substances (fat and protein) diminished as a natural result of the decreased total metabolism.

These conclusions as to the immediate effect of fasting are in harmony with those reached by Shaffer (4) in his third and fourth articles on antiketogenesis.

In contrast to the period of fasting, the subsequent period of restricted diet was accompanied in two of the patients with a very marked increase in the power to oxidize glucose. The question is, whether this might not also have taken place, had no fast been instituted. We have no data on this point. In justice it must be said, however, that without fasting many patients might fail to survive the ketosis long enough to enjoy the benefits of a restricted diet.

In spite of accumulating observations on the fall of the respiratory quotient after the ingestion of food in diabetes nothing definite is known as to its cause. McCann and Hannon (15) called attention to its occurrence after the ingestion of glucose or of glycerol. That it follows the ingestion of glucose has been noted in this laboratory in the case of Chas. S., reported in a previous paper (10). With levulose, Joslin (9) usually found an abrupt rise, but in 11 out of 58 experiments the rise was absent, and instead, a fall was noted from the end of the 3rd hour on. Wilder, Boothby, and Beeler (8) observed it after a mixed meal, especially one rich in protein and poor in carbohydrate. We have found the

depression to occur alike with the Newburgh type of diet and a balanced diet in which the three food elements were present in about equal weights. One-twelfth of the 24 hour allowance of food sufficed to cause a sharp drop. In one experiment in which a non-caloric "meal" was given, consisting of thrice cooked vegetables and seasoning, no depression occurred, although the patient had previously reacted to the ingestion of food with an unmistakable fall. Either this meal failed to stimulate the gastric secretion of HCl or else the excretion failed to affect the respiratory quotient. Curiously enough, Joslin (9) found no depression either after his one experiment with glucose, or with his numerous observations on protein-fat diets, oatmeal, or mixed diets. That the phenomenon is not confined to diabetics is shown by the tables of Benedict and Carpenter (16) which show that three out of four normal individuals reacted to glucose in the first 20 minutes by a fall in the quotient amounting to from 0.02 to 0.03. That the occurrence may be related to the "alkaline tide" is suggested by the work of Dodds (17) who found in normal individuals, after eating, a retention of CO<sub>2</sub> which he thought due to the excretion of HCl by the stomach.

#### SUMMARY AND CONCLUSIONS.

1. The fatty acid-glucose ratio, calculated from the data obtained with the respiration calorimeter, was parallel with the excretion of the acetone bodies. The threshold of ketosis was found near a ratio of 1.5 to 1. This ratio corresponds theoretically to the presence, during oxidation, of 1 molecule of keto-acid for every molecule of glucose.

2. This parallelism is evidence that the respiratory quotient in diabetes is a true measure of oxidation.

3. Diabetic patients were often able to take, without undergoing ketosis, diets containing a larger proportion of ketogenic substances than is called for by the ratio of 1 molecule of keto-acid to 1 of glucose. This was because the proportions of food-stuffs oxidized were not the same as those ingested.

4. The fall of ketosis observed in diabetic patients as a result of fasting is accounted for by the simultaneous drop in the fatty acid-glucose ratio calculated from the calorimeter data. It was due primarily to a decrease in the total metabolism with a consequent diminution in the metabolism of fat and protein.

5. At the time of fasting there was little or no increase in the total quantity of glucose oxidized, when this was computed as being derived from protein and the glycerol of the fat, as well as from the carbohydrate, undergoing metabolism during the period. Fasting reduces the blood sugar chiefly by depleting the endogenous sources of glucose. There is often an increase in the oxidation of glucose during the subsequent period of restricted diet, but this is not necessarily due to the fast.

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*Addendum*—In using the Van Slyke method for the determination of the acetone bodies 1 mg. of precipitate was taken as the equivalent of  $\frac{1}{8}$  mg. of acetone.

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